

THESIS

MUTATIONAL ANALYSIS OF THE HUMAN HISTONE CHAPERONE, NAP1, IN  
NUCLEOSOME DISASSEMBLY AT THE HTLV-1 PROMOTER

Submitted by

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## ABSTRACT OF THESIS

### MUTATIONAL ANALYSIS OF THE HUMAN HISTONE CHAPERONE, NAP1, IN NUCLEOSOME DISASSEMBLY OF THE HTLV-1 PROMOTER

The human genome is packaged to fit within the confines of the nucleus through the interaction with four core histone proteins, H2A, H2B, H3 and H4. These proteins organize the genetic material, however they also make it difficult for the cells to access the information stored within the DNA sequence for processes such as transcription and replication.

One of the mechanisms by which the genetic information can be accessed is post-translational modifications of the histone tails. Post-translational modifications, such as acetylation, act to neutralize charges on the histone tails and also serve to create new binding sites for other proteins. These modifications have been associated with decompaction of condensed chromatin, alteration of nucleosome structure, and partial or complete disassembly of the histone octamer. Our laboratory uses human T-cell leukemia virus, type 1 (HTLV-1) as a model for studying eukaryotic transcription activation and gene regulation.

Previous studies using chromatin immunoprecipitation to look at the HTLV-1 promoter have correlated transcription activation with a decrease in post-

translational modifications that are traditionally associated with gene activation. This decrease in activating modifications was due to a decrease in histone occupancy at the promoter in vivo. To recapitulate the results observed in vivo, we developed an immobilized template assay using the biotin labeled HTLV-1 promoter fragment bound to a magnetic streptavidin coupled bead. Nucleosome disassembly at the HTLV-1 promoter is dependent on the presence of the virally encoded Tax protein, as well as the phosphorylated form of the cellular protein cyclic-AMP response element binding protein (pCREB), cellular coactivators CREB binding protein (CBP)/p300, acetyl coenzyme A (acetyl CoA), acceptor DNA and nucleosome assembly protein (Nap1). Tax and pCREB recruit the histone acetyltransferase, CBP/p300, which acetylates histone tails prior to disassembly of the octamer. Nap1 is unique in this reaction because this was the first example of a histone chaperone supporting disassembly of the entire octamer in an acetyl CoA dependent fashion, independent of ATP consumption or the presence of chromatin remodeling complexes.

In this study we examined the domains of Nap1 required for in vitro nucleosome disassembly at the HTLV-1 promoter template through a series of rationally designed deletion mutants. Crystal structures of yeast Nap1 and SET/TAF-I $\beta$  were used as models for designing mutations in the human Nap1 protein. Our results show that the minimal domain of human Nap1 able to support nucleosome disassembly is contained within amino acid residues 196-290. Using histone binding assays, we also found that the ability to disassemble nucleosomes is independent of histone interaction in vitro. Removal of the  $\beta$ -

hairpin that is required for Nap1 oligomerization renders the protein unable to support disassembly. This suggests that the oligomeric form of Nap1 is required for nucleosome disassembly at the HTLV-1 promoter.

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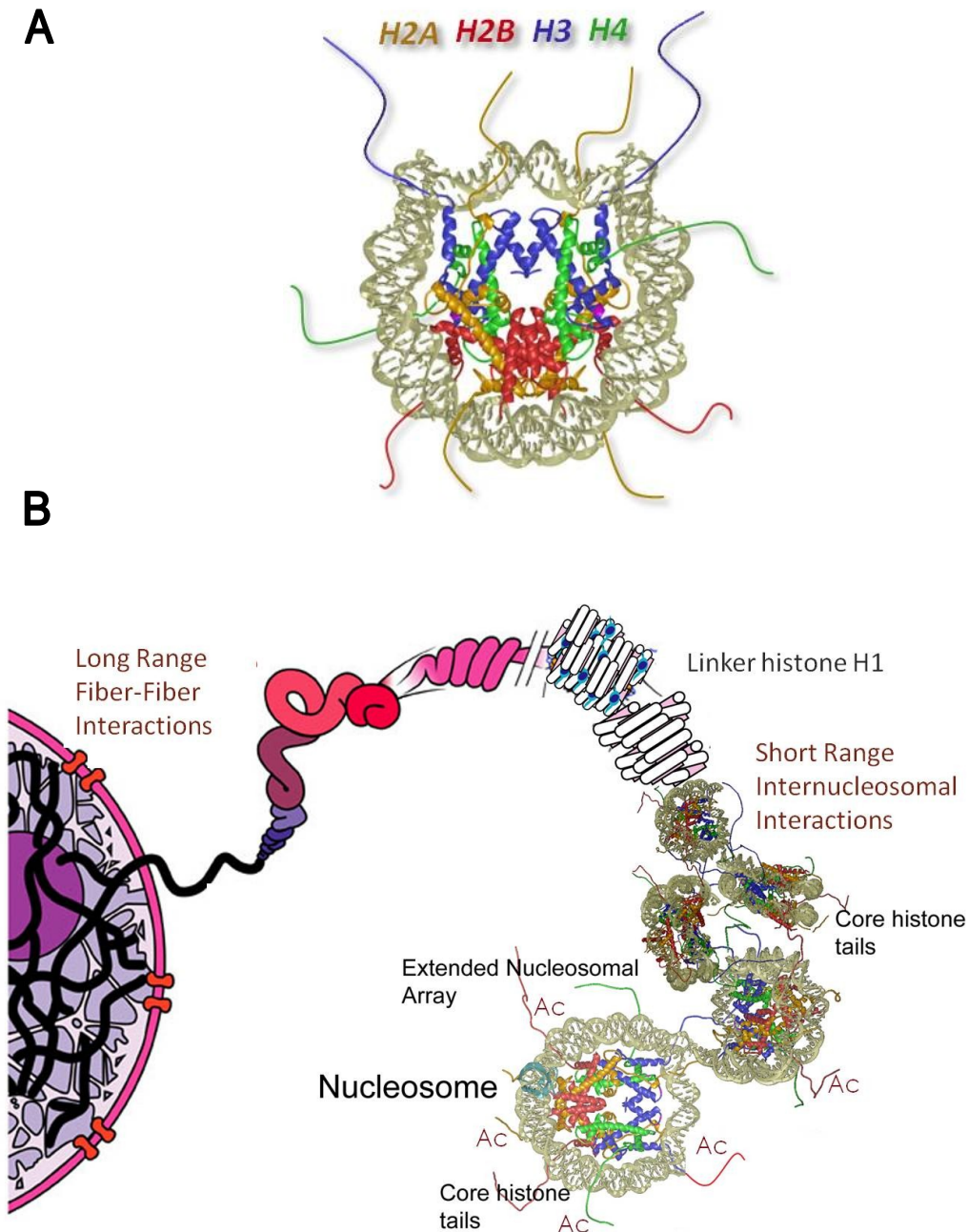


# **CHAPTER 1**

## **INTRODUCTION TO TRANSCRIPTION REGULATION AND THE HTLV-1 PROMOTER**

### **1.1 Chromatin Structure**

Within the eukaryotic cell, genetic material is packaged into chromatin through interaction with four core histone proteins. The first level of chromatin organization is accomplished by wrapping approximately 147 base pairs (bp) of DNA around an octamer containing two copies of each of the four core histone proteins, H2A, H2B, H3 and H4, to form a nucleosome (Figure 1A) [1-3]. Each histone is comprised of two parts: a highly conserved histone fold domain and an unstructured histone tail that extends beyond the gyres of the nucleosomal DNA [4]. The nucleosome assembly pathway begins with deposition of a H3/H4 tetramer onto the DNA followed by the stepwise addition of two H2A/H2B dimers. H2A/H2B dimers do not physically interact with each other because they are positioned on either side of the H3/H4 tetramer, interacting along the dimer/tetramer interface as well as with approximately 30 bp of DNA per H2A/H2B dimer. The octamer has 2-fold symmetry and wraps DNA in a left-handed coil 1.7 turns around the histone octamer to form a nucleosome. In this open form, the chromatin is referred to as a “beads-on-a-string” array because of the appearance of nucleosomal beads connected by a linker DNA string. Further



**Figure 1: Nucleosomes are the most basic unit of chromatin** (A) Crystal structure of the nucleosome showing the putative positions and orientations of the unstructured histone tails [1]. (B) Proposed model for the condensation of chromatin. Starting with the 10 nm beads-on-a-string extended array, short range internucleosomal interactions along with the addition of linker histone H1 form a condensed 30 nm fiber. Further condensation of the 30 nm fiber through long range fiber-fiber interactions condenses the chromatin to fit in the nucleus (Adapted from [7]).

compaction is achieved through addition of the DNA binding protein, linker histone H1, which interacts with the linker DNA at the entry and exit points of the nucleosome. Histone tail and fiber-fiber interactions help condense the chromatin to form a 30 nm fiber [5-7]. Finally, after a hierarchy of folding, which is not yet understood, the DNA reaches its most compacted form and the genetic material appears as chromosomes visible within the nucleus of eukaryotic cells during division (Figure 1B).

## **1.2 Transcription Activation and the Dynamic Nature of Chromatin Assembly and Disassembly**

Although the compaction of chromatin allows DNA to be condensed to fit within the confines of the nucleus while maintaining organization of genetic material, it also poses a problem for accessing the information stored in the sequence of the DNA. Hence, chromatin structure must remain dynamic, with a balance between assembly and disassembly of nucleosomes to allow the genetic material to be accessed without becoming tangled or disorganized. Several in vitro studies have shown that chromatin is repressive to transcription compared to naked DNA [8-12]. Removal of nucleosomes by sliding or disassembly is necessary when access to genetic information is required, and the DNA must be repackaged once transcription or replication is completed to maintain organization within the nucleus.

For the cell to carry out transcription, replication, and repair, a mechanism must be in place to expose the nucleosomal DNA. As a result, chromatin must, by definition, be dynamic in nature. The necessity of nucleosome removal is

demonstrated by the fact that transcription factor binding has repeatedly been shown to be inhibited by the presence of nucleosomes [13-17]. Additionally, the presence of a nucleosome at a transcription start site can physically hinder the binding of the RNA polymerase holoenzyme complex and inhibit transcription activation [18]. Removal of promoter nucleosomes allows for the binding of transcription factors and RNA polymerase II machinery as suggested by the evidence for nucleosome depleted regions at promoters of actively transcribed genes [19, 20]. In addition, studies from our laboratory of immobilized chromatin assembled promoter templates demonstrate that disassembly of promoter nucleosomes results in increases in transcription in vitro [179]. The combination of in vitro and in vivo studies mentioned above has demonstrated a correlation between depletion of nucleosomes and transcription activation. Three major ways chromatin structure can be regulated are by modification of the physiochemical properties of the chromatin (such as by charge neutralization), nucleosome mobilization by ATP-dependent remodeling complexes, and interaction with histone chaperones.

#### *Physiochemical Properties of Modified Chromatin:*

One mechanism for regulating compaction of the chromatin is through post-translational modification of the histone tails. Because the tails of the histones interact with the linker DNA between the nucleosomes, neighboring nucleosomes and long distance interactions with other histones when condensed, these unstructured tails make ideal targets for modifications that neutralize the charge and affect the chromatin structure. Post-translational

modifications of the histone tails also serve as new binding sites for modifying proteins that recognize altered residues.

#### *ATP-Dependent Remodeling Complexes:*

The interaction between histones and DNA is very stable [21, 22]. Factors such as ATP-dependent remodeling proteins affect the stability of the nucleosomes so that they can be removed when needed but not disassemble randomly (reviewed in [23]). Traditionally, these remodelers have been thought to function by disrupting histone-DNA contacts to aid in sliding of nucleosomes, however an additional role for ATP-dependent remodeling complexes in the disassembly of nucleosomes has been suggested.

#### *Histone Chaperones:*

Under normal physiological conditions, histones and DNA do not readily assemble into nucleosomes, instead they form non-specific aggregates [24]. Histone chaperones promote organized deposition of histones to form productive, stable nucleosomes. Histone chaperones are negatively charged proteins that guide the assembly process and prevent non-productive interactions between positively charged histone proteins and negatively charged DNA [67]. These proteins can assemble histones and DNA into nucleosomes or disassemble nucleosomes to their components, histones and DNA [25-29].

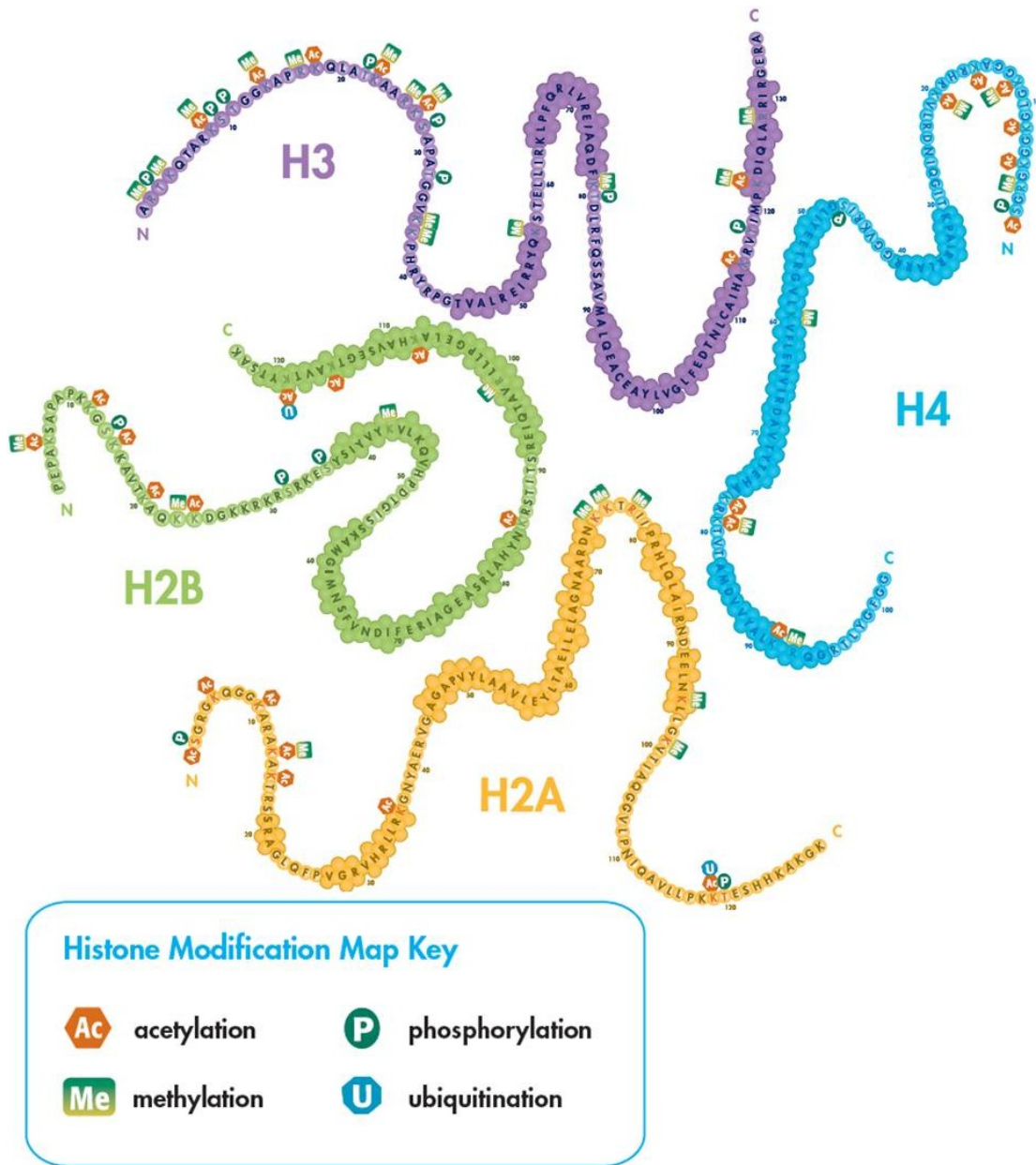
### **1.3 Gene Regulation by Post-Translational Modifications**

It has been proposed that a histone code exists in which a particular combination of histone modifications signifies a specific change in the activity or arrangement of the chromatin to participate in gene regulation [30, 31]. There

are a variety of known post-translational modifications of histones, the most common including acetylation, methylation, phosphorylation, and ubiquitination, with acetylation being the most highly studied (Figure 2) (reviewed in [32]). Modifications occur primarily on the amino terminal tails of all four core histones and may occur in multiple combinations to regulate gene expression. For example, mono-methylation of lysine residues in the H3, H4 and H2B tail is associated with activation [33-35] while di- and tri-methylation of H3K9 and H3K27 is associated with repression [33, 36]. One of the most important histone tail modifications is acetylation of lysine residues by histone acetyltransferases (HATs) because of the strong correlation between acetylation and gene activation. Acetylation of H3 K9 and K14 is associated with activation [37].

#### **1.4 Effects of Histone Acetylation on Chromatin Structure**

As stated above, lysine acetylation of the histone tails is associated with gene activation and is one of the most well characterized histone tail modifications [31, 38-40]. Acetylation is best known for its role in neutralizing the positive charges on the histone tails and therefore disrupting internucleosomal interactions, resulting in relaxation of the chromatin fiber. Multiple studies have shown that acetylation of the histone tails correlates with decondensation of compacted chromatin [41-44], along with alteration of nucleosome structure [45-48]. For example, our lab has shown that lysine acetylation at a H3K14 promotes disassembly of promoter nucleosomes [179].



**Figure 2: Sequence of the four core histones depicting the published post-translational modifications.** The bold and enlarged portions represent the alpha helices of the published crystal structure [1]. Histone Modification Map – created by Millipore Corporation ([www.histone.com/modification\\_map.htm](http://www.histone.com/modification_map.htm)).

Acetyl groups are added by histone acetyltransferases (HATs) and can be removed by histone deacetylases (HDACs). Traditionally, acetylation of the histone tails has been correlated with transcription activation and removal of acetyl groups is a mechanism for transcription repression [40]. Once acetylated, it is unknown how the presence of acetyl groups promotes gene activation, however it is known that histone acetylation increases the ability of transcription activators to bind DNA [16, 17, 49]. Additionally, the presence of an acetyl group may lead activation transcription by serving as a binding site for transcription regulatory complexes containing bromodomain subunits that recognize acetyl groups [50]. Acetyl groups may also alter the properties of chromatin and promote decondensation of compacted chromatin through weakening of interactions between histone tails and neighboring nucleosomes or linker DNA. However, acetylation has no effect on the overall stability of the nucleosome [45-48] because histone tails do not directly contribute to its stability [47, 51-53].

Recently, p300, a well-known HAT has been identified as a signature for transcriptionally active chromatin [19, 54]. The recruitment of p300 to promoters, followed by histone acetylation has been associated with transcription activation [13, 55-60]. The opposing effects of acetylation by HATs and deacetylation by HDACs help maintain a balance between transcriptionally active and transcriptionally silent chromatin.

### **1.5 Histone Chaperones and Transcription Activation**

Histone chaperones have been implicated in the assembly, disassembly, and sliding of nucleosomes [61, 62]. The four core histones have been found in



complex with histone chaperones in multiple eukaryotic species [27, 63-66]. Chaperones help prevent inappropriate interactions between histones and DNA that would disfavor in the formation of a nucleosome [67]. This is accomplished by shielding the charge of the histones by the histone chaperone until the histones are deposited onto the DNA in a functional manner [63, 68]. Once appropriate contact with the DNA is made, the histone chaperone releases the histones. Although histone chaperones are capable of assembling nucleosomes without the aid of additional proteins or consumption of energy, during the disassembly process, chaperones frequently act in conjunction with ATP-dependent remodeling proteins that use the energy from ATP hydrolysis to break histone/DNA contacts [69].

In most cases, a specific histone chaperone will interact and stabilize either H2A/H2B dimer or H3/H4 tetramer. However, there are a few examples of chaperones that are capable of interacting with all four core histone proteins, either in their dimer and tetramer forms or as a complete octamer [70-73].

### **1.6 The Histone Chaperone Nap1**

Nucleosome assembly protein 1 (Nap1) was originally identified in HeLa cells and is one of few histone chaperones that is capable of recognizing all four core histone proteins [74]. Nap1 is a member of the Nap family of histone chaperone proteins that includes SET/TAF-I $\beta$ , Vps75, as well as the human Nap1 variants Nap1-like proteins 1-5 (Nap1L1-5) and has homologues in yeast, *Drosophila*, and *Xenopus* (Figure 3). Members of the Nap family of proteins are highly conserved and have a broad range of functions including



transcription activation, assembly and disassembly of nucleosomes, and interaction with other proteins that carry out cellular processes unrelated to chromatin dynamics and transcription activation [75]. Specifically, Nap1 interacts with cellular proteins that carry out many diverse functions including cell cycle regulation, mediation of nuclear import, control of RNA synthesis and translation, ATPase activity within the mitochondria, lysine biosynthesis, and histone transport and control (reviewed in [75]). Of these functions, the most highly studied is the histone chaperone activity of Nap1 because of its relevance to transcription activation and chromatin dynamics.

In the cell, under physiological conditions, Nap exists as a dimer and does not exist in monomeric form [76]. As a chaperone, Nap1 is capable of interacting with both H2A/H2B dimers and H3/H4 tetramers. Generally the ratio of interaction is 1:1, Nap monomer:histone monomer. Specifically, one Nap dimer interacts with one H2A/H2B dimer or two Nap dimers interact with one H3/H4 tetramer [25, 73].

Histone chaperones, such as Nap1, have been implicated in maintaining a balance between assembly and disassembly of nucleosomes [62]. Previously, Nap1 was known for H2A/H2B dimer removal and exchange [61, 77-80]. However, recent evidence suggests a role for Nap1 in disassembly of all four core histones [60, 78, 81]. Of significance, the Nap1-dependent disassembly of nucleosomes from the HTLV-1 promoter was the first example of a histone chaperone functioning to disassemble the complete octamer in an acetylation-dependent manner [60]. This reaction occurs in the absence of ATP-dependent

chromatin remodeling proteins, transcription initiation, and elongation, discussed in Sharma and Nyborg 2008 [60]. This observation was noteworthy as Nap1-mediated disassembly occurs in the absence of ATP, but required acetyl coenzyme A (acetyl CoA) and the HAT activity of p300.

### **1.7 Brief History of HTLV-1 and its Infectivity**

Discovered in 1980, human T-cell leukemia virus type-1 (HTLV-1) was the first characterized human retrovirus [82]. It is estimated that between 10 and 20 million people are infected with HTLV-1 worldwide, with high incidence in southwest Japan, the Caribbean, and parts of central Africa and South America [83-88]. The virus is the causative agent of the aggressive malignancy, adult T-cell leukemia/lymphoma (ATLL), as well as the neurodegenerative disease tropical spastic paraparesis/HTLV-1 associated myelopathy (TSP/HAM) (reviewed in [89]). The virus can be transmitted from person to person by exposure to contaminated blood, blood transfusions, sexual contact, or from mother to child by breastfeeding [83-88]. Once infected, there is a prolonged period of viral persistence in which the majority of individuals remain lifelong asymptomatic carriers of the virus.

### **1.8 HTLV-1 as a Model System for Studying Nucleosome Disassembly and Transcription Activation**

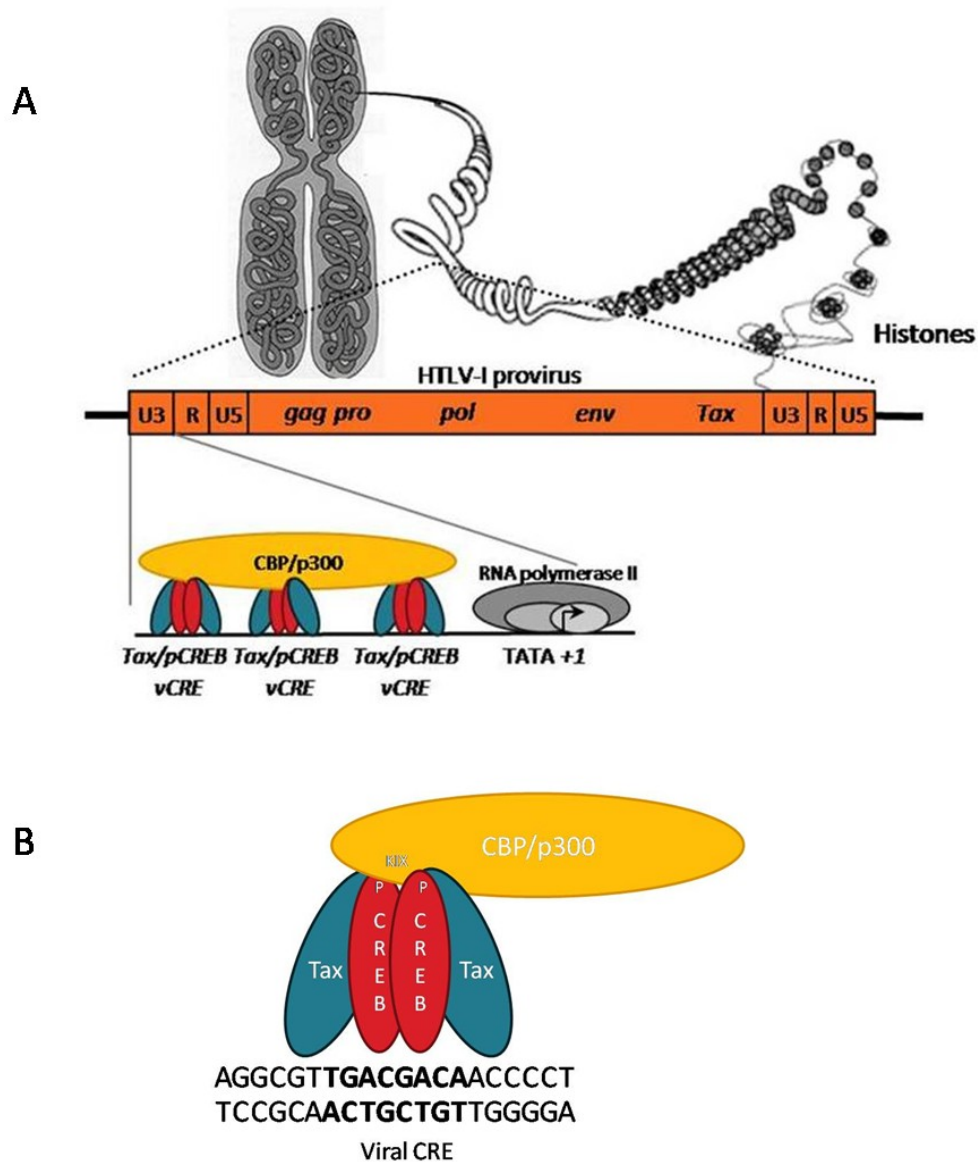
The HTLV-1 promoter is useful for studying eukaryotic transcription and gene expression because the provirus becomes stably and permanently integrated into the host cell's chromosomes. Once integrated, the provirus is packaged into chromatin in the same manner as a cellular gene. In the context

of the chromosome, the viral DNA is indistinguishable from cellular genes and is regulated similarly, utilizing cellular transcription activators, coactivators, and polymerase machinery (Figure 4A). In addition, HTLV-1 encodes its own potent transcription activator, Tax, which activates transcription more than a thousand fold in vivo [90].

Previously, HTLV-1 has been used as a model promoter for both in vitro and in vivo studies of transcription activation in a chromatin context [13, 60, 90, 179]. This promoter is useful because it has a relatively small regulatory region that spans less than 300 base pairs rather than thousands of base pairs often observed for cellular gene promoters (discussed in more detail in the following section).

### ***1.8a HTLV-1 Provirus and Promoter***

Like other retroviruses, HTLV-1 encodes its own structural proteins, transmembrane proteins, and surface glycoproteins along with reverse transcriptase and integrase. Importantly, the viral genome also has regulatory and accessory proteins encoded in the pX region including Tax [91, 92] and Rex [93]. Tax will be described in greater detail in the following section. Flanking the protein encoding region are two identical long terminal repeats (LTRs) characteristic of all retroviruses [94-97]. Each LTR is identical and is subdivided into three parts; the unique 3' (U3), the repeated (R), and the unique 5' (U5) region. The U3 region is critical for our studies of transcription activation because it contains the HTLV-1 promoter elements and is followed immediately by the transcription start site at the U3/R junction [94-97] (Figure 4A).



**Figure 4: Protein interactions at the integrated HTLV-1 provirus** (A) The HTLV-1 provirus has two long terminal repeats, each containing a U3, R, and U5 region as well as coding regions. The provirus is integrated into the host cell's chromosome and is packaged into chromatin in the same manner as the cellular genetic material. U3 is the primary regulatory region and contains three viral CREs that act to recruit activators and coactivators and a TATA element. The transcription start site is at the U3/R junction. (B) The phosphorylated form of the cellular transcription activator CREB binds as a dimer to the viral CRE to an 8 bp consensus CRE sequence. The viral oncoprotein, Tax interacts with CREB and the GC-rich regions that flank the viral CRE and serves to recruit the ubiquitous coactivator p300.

Following infection, the viral RNA genome is reverse transcribed to make a double-stranded DNA copy of the viral genome. Upon completion, the viral protein integrase randomly clips the host's DNA, inserts the viral sequence, and repairs the breaks [98, 99]. Once integrated, the viral genome is packaged into chromatin. Regulation of viral transcription (i.e. replication) is regulated by a combination of cellular and viral transcription activators and coactivators.

In the U3 promoter region within the LTR there are three highly conserved 21 bp repeats known as viral cyclic AMP response elements (vCREs) , located at -100, -200, and -250 bases relative to the transcription start site at +1 [100-103]. These elements are required for Tax-activated transcription and are named so because their 8 bp core sequences resemble those of cellular enhancers known as CREs.

### ***1.8b CREB and Tax Recruitment***

Initiation of viral transcription begins with the recruitment of transcription activators to the viral CREs. Normally, cellular CREs and bind the cellular transcription activator CREB (cyclic-Amp response element binding protein) or other ATF/CREB family members [104-110]. To activate HTLV-1 through the viral CRE, CREB must become phosphorylated at ser133 through a pathway induced by the viral protein, Tax [111, 112]. Phosphorylated CREB (pCREB) binds as a dimer to the central 8 bp of the vCRE. Binding of pCREB is followed by the interaction of two Tax molecules that form contacts with either side of the pCREB dimer [113]. Tax also makes protein-DNA contacts with the GC-rich regions of the vCRE that flank the 8 bp element (Figure 4B) [114-117]. The

requirement for phosphorylated CREB rather than unphosphorylated CREB for complex formation with Tax at the HTLV-1 vCRE is consistent with the observation of elevated pCREB levels in Tax-expressing cells [112, 118, 119]. Tax cannot bind to the vCRE in the absence of CREB [117], however by virtue of the pCREB/Tax interaction, Tax is able to bind the vCRE and at the same time Tax increases the binding affinity of pCREB for the DNA [106, 109, 110, 120-123].

### ***1.8c Coactivator Recruitment to the HTLV-1 Promoter***

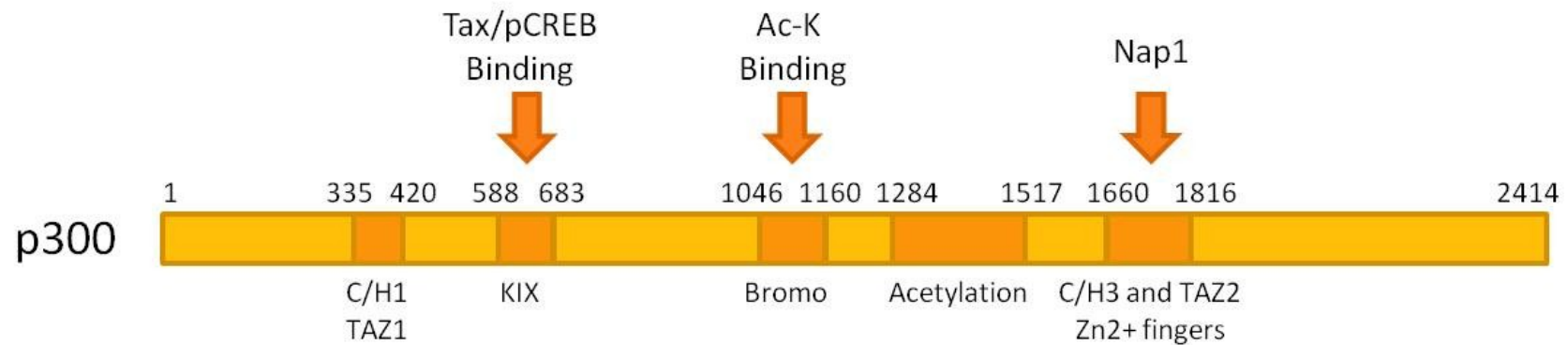
Tax binding to the pCREB/vCRE complex is required for high levels of transcription of the HTLV-1 provirus [100, 101, 103, 124]. As mentioned above, Tax and pCREB form a stable complex on the vCRE enhancer elements. This complex, although required, is not sufficient for gene activation. Together, pCREB and Tax recruit the cellular coactivator and histone acetyltransferase p300 or its homolog CREB binding protein (CBP) [57, 125, 126]. At the HTLV-1 promoter, both CBP and p300 are functionally equivalent, and therefore will be referred to as CBP/p300. Tax, pCREB, CBP/p300, and the vCRE form a stable quaternary complex through interaction of CBP/p300's KIX domain with Tax and pCREB [13, 58, 112, 125-128]. Full length p300 has been shown to be recruited to the HTLV-1 by the Tax/pCREB complex both in vitro and in vivo [57, 58, 60, 90, 129-131]. Importantly, Tax recruitment of CBP/p300 promotes histone acetylation. The phosphorylated form of CREB in complex with Tax is absolutely required for recruitment of the coactivators CBP/p300 to activate transcription of the HTLV-1 provirus [132-135]. In addition to interactions between Tax and



pCREB, the Tax, pCREB, vCRE complex interaction is stabilized greatly through interaction with CBP/p300 (Figure 4B) [125-128, 136]. Following the stable recruitment of CBP/p300 to the HTLV-1 promoter, the intrinsic HAT activity of the coactivators results in acetylation of the core histone tails of promoter nucleosomes, a critical step in the gene activation process.

#### ***1.8d The Cellular Coactivator p300 and the Histone Chaperone Nap1***

At 2441 and 2414 amino acids in length respectively, CBP and p300 are very large and extremely homologous proteins that show significant evolutionary conservation in both sequence and domain function (Figure 5) [137]. These cellular coactivators carry a histone acetyltransferase domain and are known to acetylate lysine residues in all four core histones [138, 139], both free in solution and in complex with DNA as nucleosomes [139-142]. This HAT activity is the primary means by which these proteins regulate gene expression [140, 142]. Additionally, binding of CBP and p300 has been detected at the transcription start and end sites at more than 16,000 genes in the human genome [143], with enrichment at many active promoters [19]. Further, chromatin immunoprecipitation (ChIP) studies have shown that there is a correlation between CBP/p300 binding to the promoter and histone acetylation [90, 130, 131]. Recruitment of CBP/p300 to promoters and enhancers correlates with acetylation of histone H3 and H4 tails [140-142, 144-147]. CBP/p300 do not interact directly with promoter DNA; in order for CBP/p300 to function, they must be recruited by DNA-bound factors, such as the recruitment of CBP/p300 to the HTLV-1 promoter via the vCRE bound Tax-pCREB complex.



**Figure 5: Linear schematic of p300.** Nap1 has been shown to interact with p300 through the C/H3 domain of p300. p300 is a very large protein that has many interacting domains including the KIX domain that interacts with Tax and pCREB, the Bromodomain that has been shown to bind acetyl-lysine residues, and the histone acetyltransferase domain. CBP has the same domain layout as p300 but with slight differences in the specific beginning and ending amino acid of each domain. In addition to the interactions shown, which are pertinent to HTLV-1, many other proteins interact with both CBP and p300. (Adapted from [138]).

In 2006, Lemasson et al detected that upon Tax expression, pCREB and p300 were recruited to the HTLV-1 promoter using the chromatin ChIP assay. Unexpectedly, they also detected decrease in the activating post-translational modifications, acetylation and methylation, of the histone tails. Consequently, the decrease in these activating modifications was attributed to depletion of histone H3 and linker histone H1 at the HTLV-1 promoter [90].

To recapitulate results from in vivo studies our laboratory initiated in vitro immobilized template studies using a fragment containing the HTLV-1 promoter assembled into chromatin. We found that following the addition of purified Tax, pCREB, p300, acceptor DNA, acetyl CoA, and the histone chaperone Nap1, nucleosomes were disassembled from the HTLV-1 promoter [60]. Importantly, this reaction was performed in the absence of ATP and chromatin remodeling proteins but required the histone chaperone Nap1. In this reaction, Nap1 was the most difficult protein to identify, as it was originally supplied through the chromatin assembly process.

### ***1.8e The Interconnection between Nap1, HATs and Nucleosome Mobilization***

There are many individual links between histone chaperones, histone acetyltransferases, and nucleosome disassembly. The following is a bulleted list of the relevant links that establish a foundation for the study of Nap in acetylation-dependent nucleosome disassembly.

- Nucleosome depleted regions are observed in yeast, fly and humans near the start sites of transcription, and the level of depletion is directly related to the level of gene expression [19].
- There is a level of p300 enrichment at many active promoters [19].
- Stable complexes with Nap1, p300, and H2A/H2B dimer have been identified [59, 148, 149].
- During transcription the displacement of histones is enhanced by Nap1 [150].
- Acidic histone binding proteins such as the histone chaperone SET/TAF-I $\beta$  have been linked to assembly and disassembly of H2A/H2B [80].
- SET/TAF-I $\beta$  is also a potent activator of transcription through its association with chromatin [151].
- FACT (facilitator of activated chromatin transcription), another histone chaperone, binds nucleosomes and displaces one or both dimers in transcribed regions [152, 153].
- The chaperone nucleoplasmin assembles and disassembles nucleosomes in the remodeling of sperm chromatin [154].
- In yeast, cells deficient for Nap1 have more than a two-fold change in the expression pattern in 10% of genes [155].

Our in vitro, biochemically defined system is unique because it is the first example of Nap1 disassembling nucleosomes from a natural promoter in an acetylation-dependent fashion that is independent of ATP consumption [60]. In

support of the findings of nucleosome disassembly at the HTLV-1 promoter, Nap1 has been shown to stably interact with p300 [59, 148, 149].

Nap1 is utilized by multiple viruses to bind viral and cellular proteins to promote viral transcription [60, 149, 156-158]. Additionally, Nap family members have frequently been shown to act in conjunction with other viral systems. Specifically, SET/TAF-I $\beta$  has been shown to stimulate chromatin decondensation [159], interact with CPB/p300 to modulate acetylation [160], and activate adenovirus transcription [161]. In addition, Nap1 has been shown to function at other viral promoters to aid in transcription activation. Nap1 interacts with the HIV Tat protein and promotes viral transcription [158], and a direct interaction between Nap1 and E2 protein has been shown to activate Papillomavirus transcription [156].

The research discussed above sets a precedent for the link between histone chaperones of the Nap family, interaction with CBP/p300, and activation of viral transcription. The retroviral model is useful for studying eukaryotic gene regulation because Nap1-mediated disassembly occurs at viral promoters that are packaged into chromatin, indistinguishable from cellular genes.

### ***1.9 Nap1-Dependent Nucleosome Disassembly at the HTLV-1 Promoter***

To summarize, disassembly of HTLV-1 promoter nucleosomes begins with the recruitment of Tax and pCREB. The cellular protein, CREB becomes phosphorylated by a pathway induced by Tax. Tax is a virally encoded oncoprotein known to stimulate HTLV-1 transcription. Tax, pCREB, and p300 form a stable quaternary complex with the HTLV-1 promoter DNA. Nucleosome

disassembly at the HTLV-1 promoter also requires acetyl CoA as a substrate for p300's HAT activity and the histone chaperone Nap1. HTLV-1 is a useful tool for studying eviction of histones because a highly purified biochemically defined system can be used to monitor disassembly uncoupled from transcription using purified activators and coactivators. Work from our laboratory has demonstrated that Tax, pCREB, p300, acetyl CoA, acceptor DNA and Nap1 are absolutely required for nucleosome disassembly from the HTLV-1 promoter.

Although disassembly is dependent on Nap1, the mechanism by which this histone chaperone disassembles nucleosomes is unknown. For my thesis research I set out to use mutational analysis to characterize the minimal domain of Nap1 required for disassembly of nucleosomes from the HTLV-1.

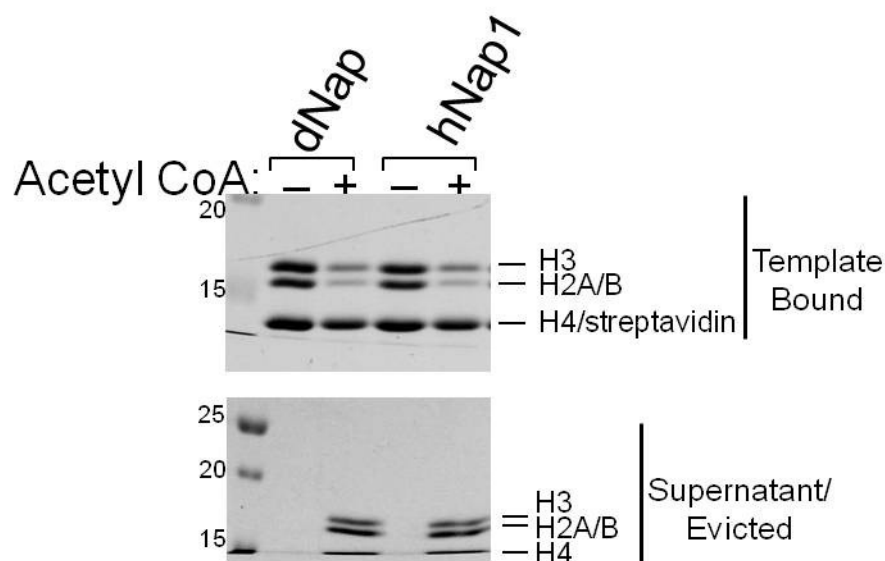
## **CHAPTER 2**

### **DESIGN AND CLONING OF NAP1 DELETION MUTANTS: DETERMINING THE MINIMAL DOMAIN OF HUMAN NAP1 REQUIRED FOR NUCLEOSOME DISASSEMBLY**

#### **2.1 Background**

Nucleosome disassembly from the HTLV-1 promoter is dependent on Tax, pCREB, Nap1, p300, acetyl CoA, and acceptor DNA [60]. Interestingly, eviction of the octamer is independent of chromatin remodeling complexes that utilize ATP. Instead, our data points to a prominent role for Nap1 in facilitating disassembly of the acetylated nucleosomes. Because of the novelty of Nap1 in acetylation dependent nucleosome disassembly, I focused my studies on Nap1 and its physical properties to aid in determining its role in nucleosome disassembly from the HTLV-1 promoter.

The original studies published by Sharma and Nyborg in 2008 used full length *Drosophila* Nap1. Recognizing that all the other non-histone proteins used in the assay were of human origin or derived from a human retrovirus, we incorporated human Nap1 (hNap1) into the histone eviction assays [60]. We found that hNap1 performed indistinguishably from dNap1 in the eviction assays (Figure 6).

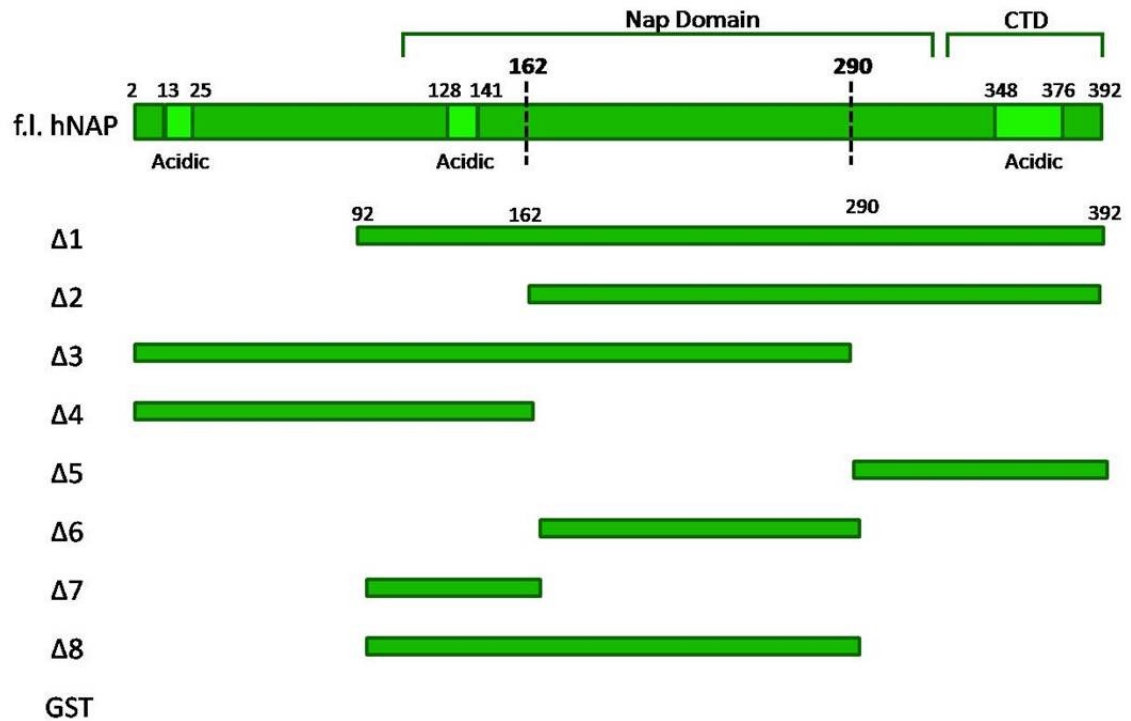


**Figure 6: Drosophila and human Nap are function indistinguishably in the acetyl CoA-dependent disassembly of HTLV-1 promoter nucleosomes.** Using the same protocol shown in figure 18, disassembly was assayed at the HTLV-1 chromatin assembled promoter following addition of Tax, pCREB, p300, and acceptor DNA. Nucleosome disassembly required the presence of acetyl CoA, as indicated. Histone proteins present in the template-bound and supernatant (evicted) fractions were detected by SDS-PAGE followed by Coomassie staining. Experiment performed by Neelam Sharma.

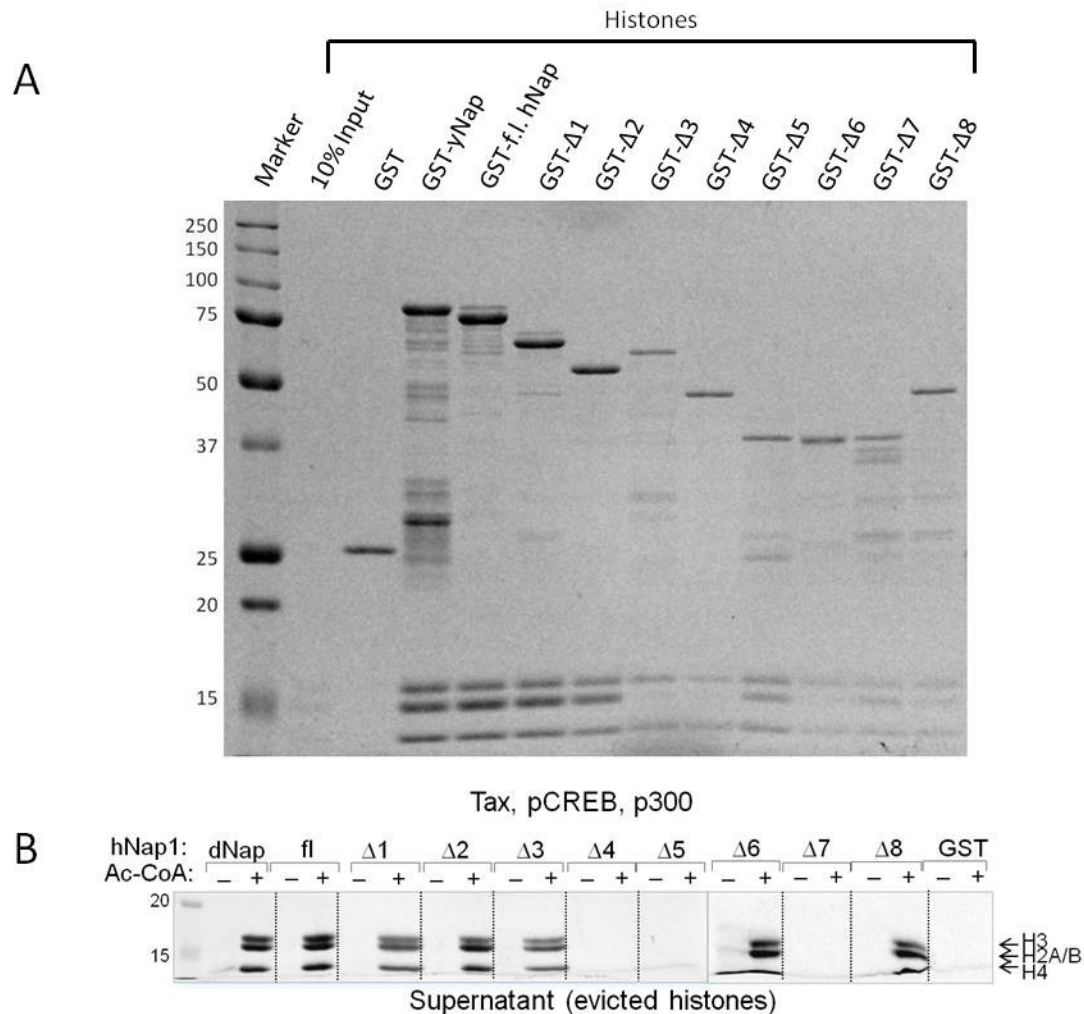


Using the human Nap1 protein, we were interested in determining the minimal domain of hNap1 required for nucleosome disassembly at the HTLV-1 promoter. Further studies were performed in our laboratory using deletion mutants of hNap1 obtained from the Giacca laboratory, which were originally cloned the Steger laboratory [156] (see Figure 7 for deletion locations). Based on the sequence alignment with yeast Nap1, the hNap1 deletions were designed to occur at the domain junctions determined by the yeast Nap1 crystal structure. These hNap1 deletion mutants allowed for a comprehensive analysis of the domains of hNap1. GST pull-downs were used to examine histone binding capability, and eviction assays were used to test the disassembly function of the mutants (Figure 8). Binding studies were used to determine if a correlation exists between hNap1-histone interaction and the ability to disassemble nucleosomes from the HTLV-1 promoter (Figure 8A). All hNap1 domains containing residues 162-290 support disassembly of nucleosomes from the chromatin-assembled HTLV-1 promoter template (Figure 8B). This particular domain is referred to as hNap1 $\Delta$ 6 by Rehtanz et al 2004 and will be referred to as such throughout this report.

The goal of the work discussed in this chapter is to design and generate further hNap1 $\Delta$ 6 deletion mutants to be tested in GST pull-downs and for their ability to support nucleosome disassembly. Although no structure of hNap1 is currently available, we designed the mutants using a computer-generated model to limit the impact on the structure as much as possible, discussed in more detail in the results section.



**Figure 7: Linear diagram of full length human Nap1 and deletion mutants.** Deletion mutants of full length human Nap1 enable a comprehensive analysis of domains required for nucleosome disassembly from the HTLV-1 promoter. Truncation locations were designed using sequence alignment of human Nap1 and yeast Nap1 and the domains in the crystal structure of yeast Nap1 (Adapted from [156])



**Figure 8: Functional analysis of full length hNap1 and various Nap1 deletion mutants** (A) GST pull-down assay indicates that the Nap1 deletion mutants have variable capacity to bind histones. GST pull-down was performed with 20 pmol GST-tagged yeast Nap1 (yNap1), hNap1, or the indicated hNap1 deletion mutant (see figure 8). GST-hNap1 proteins were incubated with 10 pmol histone octamer as describe in Chapter 3. Bound proteins were analyzed by SDS-PAGE followed by Coomassie staining. Experiment performed by Dinaida Egan. (B) Nucleosome eviction assay reveals that hNap1 proteins carrying the core region of the Nap domain (see figure 7) support nucleosome eviction. The hNap1 deletion mutant composed of amino acids 162-290 (hNap1Δ6) represented the minimal region required for nucleosome disassembly and thus served as the parent construct for further analysis. Experiment performed by Neelam Sharma

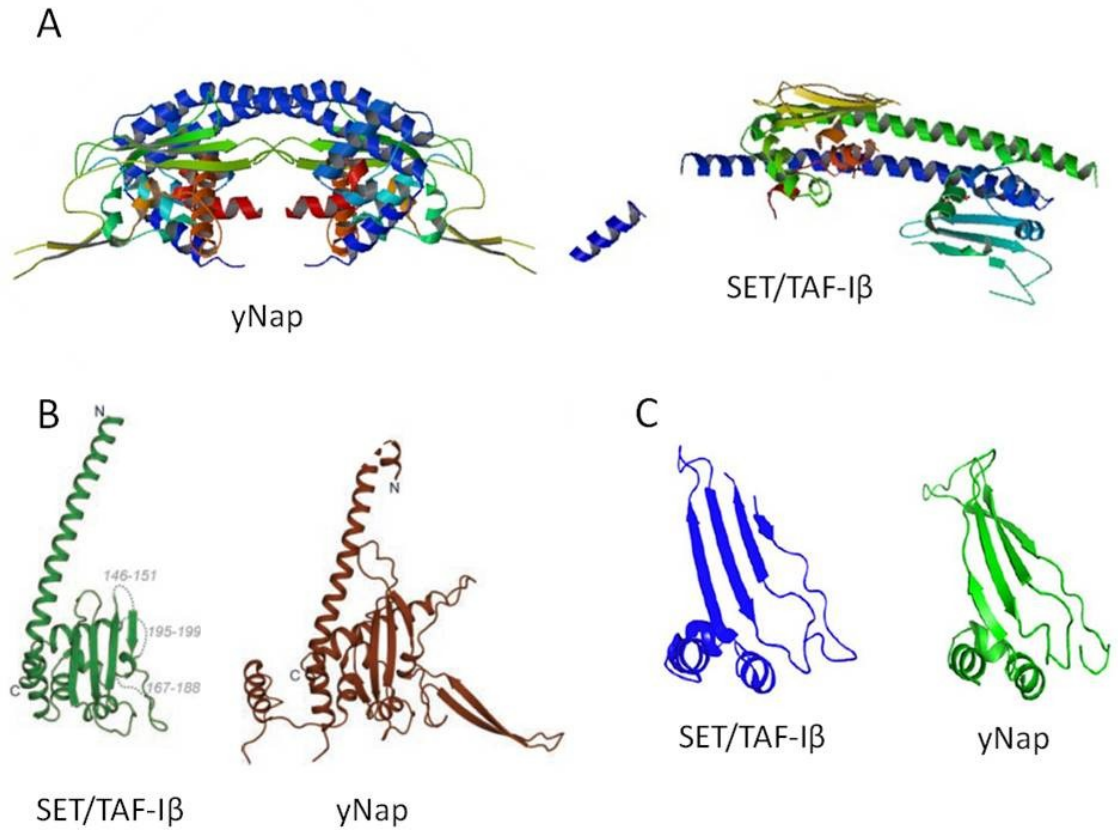
To aid in designing mutants, we utilized the crystal structures available for two other conserved Nap family histone chaperone members, yeast Nap and human SET/TAF-I $\beta$  to predict the structure of hNap1. There is significant structural and sequence homology between the histone interacting domains of hNap1, SET/TAF-I $\beta$ , and yNap1 [162] (Figure 9). All three proteins form homodimers and have similar structural characteristics (Figure 9A). Both yNap and SET/TAF-I $\beta$  have a long  $\alpha$ -helix at the N-terminus that serves as a dimerization domain followed by a globular region comprised of three more separate domains (Figure 9B). One domain of the globular region has been shown to bind histones. This domain is referred to as the “green” domain of yNap1 [163], the “earmuff domain” of SET/TAF-I $\beta$  [160], or  $\Delta 6$  of hNap1 (Figure 9C).

Based on the results of the structural prediction software, I created a series of rationally designed mutations that remove either the N- or C- terminals from hNap1 $\Delta 6$ . The work discussed in the chapter outlines the design, cloning, and purification of these deletion mutations.

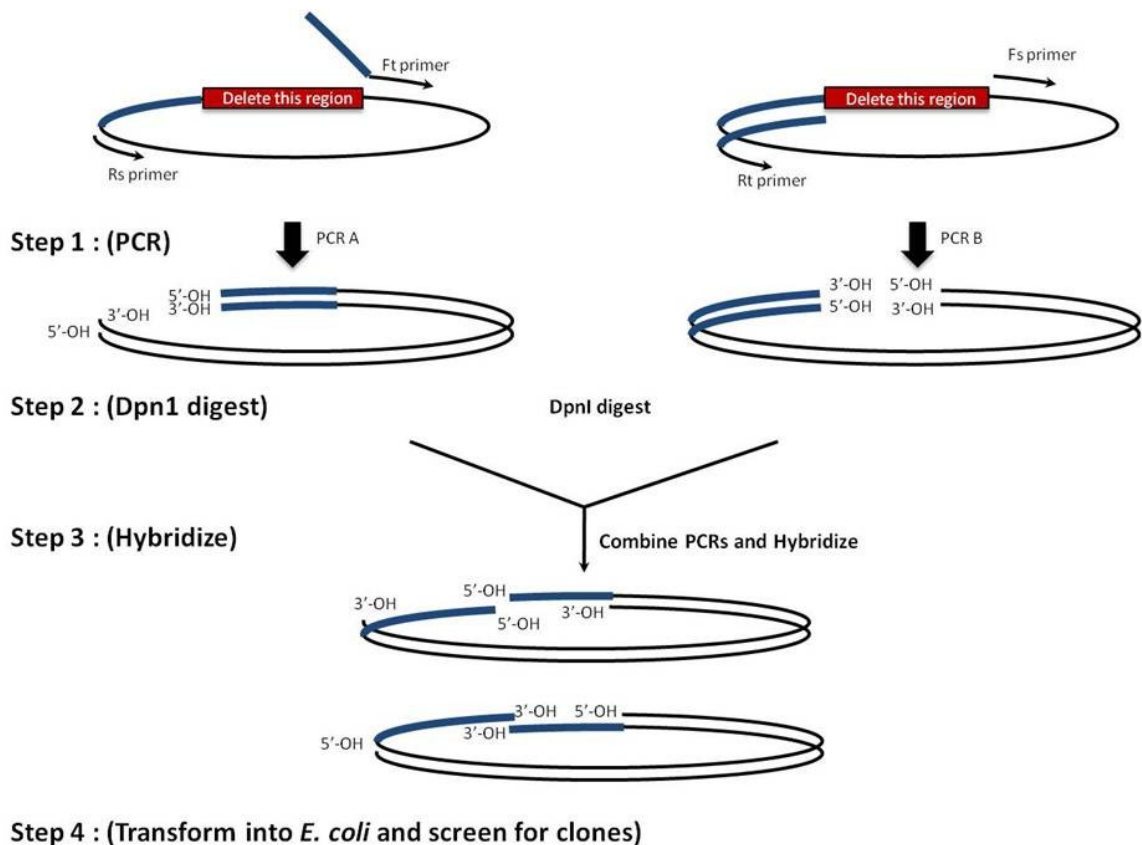
## **2.2 Materials and Methods**

### **SLIM cloning**

Site-directed ligase-independent mutagenesis (SLIM) procedure was adapted from [164, 165]. SLIM can be used for generating insertions, deletions, and point mutations. Figure 10 shows the reaction set-up for generating deletion mutants.



**Figure 9: Comparison of crystal structures of yeast Nap1 and human SET/TAF-1 $\beta$**  (A) Crystal structures of yeast Nap and SET/TAF-I $\beta$  dimers [162, 163] (B) Single monomer of SET/TAF-I $\beta$  and yeast Nap to show similar domain layout. Adapted from [178]. (C) Structural comparison of the central histone binding domain of human SET/TAF-I $\beta$ .



**Figure 10: Site-directed ligase-independent mutagenesis reaction example.** SLIM can be used for making deletions and insertions. Each mutant requires 4 primers. A combination of deletions and insertions can be made using a single set of primers. Two polymerase chain reactions are done. A short forward primer is paired with a reverse long primer and vice versa. (F and R stand for Forward and Reverse, S and L stand for Short and Long). PCR is performed and template is digested with Dpn1. The reactions are hybridized and transformed into *E. coli*. Following transformation positive clones are screened by colony PCR.

To make a deletion mutant, primers are designed to flank the sequence that will be deleted. Four primers were designed per mutant: forward primer with and without a tail and reverse primer with and without a tail. Each mutant requires two separate PCR reactions. Two primers are used per reaction. A forward tailed primer is paired with a reverse un-tailed primer and vice versa. Reactions contained 2 mM dNTP mix, 50 ng pGEX-2T Nap1Δ6 plasmid template, 10 pmol of each primer, 1xPhusion HF-Buffer, and water to a final volume of 25 μL. Samples were incubated at 98°C for 2 min prior to addition of polymerase and the temperature was reduced to 85°C for polymerase addition (0.5 U Finnzymes Phusion High-Fidelity DNA Polymerase, NEB Product Code F-530). The reaction was allowed to proceed for 40 cycles (95°C for 15 sec, 55°C for 20 sec, 68°C for 3.5 min) followed by 68°C for 7 min and a 4°C hold. After amplification, samples were diluted 5 fold in D-buffer (5XD-Buffer: 20 mM MgCl<sub>2</sub>, 20 mM Tris pH 8.0, 5 mM DTT) and digested with 0.5 U DpnI (NEB Catalog #R0176) for 1 hour at 37°C to digest template DNA.

After digestion, samples were hybridized by combining each amplification reaction along in H-buffer (3XH-Buffer: 750 mM NaCl, 125 mM Tris pH 9.0, and 100 mM EDTA pH 8.0). Reactions were heated to 99°C for 3 min to inactivate the enzyme and subjected to three cycles of 65°C for 5 min and 30°C for 15 min. The entire hybridization reaction was transformed into DH5α cells and amplified on LB agar plates containing ampicillin overnight at 37°C. Colonies were screened by PCR for successful mutation. A primer was designed to be unique to the deleted sequence. Lack of amplification product was an indication of

successful cloning as determined by ethidium bromide stained 1% agarose gel. Mutant plasmid containing cells were expanded and the plasmid was purified.

Primers for generating hNap1 $\Delta$ 6 $\Delta$ N:

*F<sub>T</sub>*: 5' CTG GTT CCG CGT GGA TCC TTG AAA GAT AAT AAA GTG AAG TTC TCA GAT 3'

*F<sub>S</sub>*: 5' TTG AAA GAT AAT AAA GTG AAG TTC TCA GAT GCT GCC C 3'

*R<sub>T</sub>*: 5' GGA TCC ACG CGG AAC CAG ATC CGA TT TGG TGG ATC GTC 3'

*R<sub>S</sub>*: 5' ATC CGA TTT TGG AGG ATG CTG GCC ACC 3'

Primers for generating hNap1 $\Delta$ 6 $\Delta$ C:

*F<sub>T</sub>*: 5' TAC CAG ATA GAT TGG AAA GGA TCC CCG GGA ATT CAT CGT 3'

*F<sub>S</sub>*: 5' GGA TCC CCG GGA ATT CAT CGT 3'

*R<sub>T</sub>*: 5' TTT CCA ATC TAT CTG GCA CCC TGT ACA ACC CAT AAT TTC 3'

*R<sub>S</sub>*: 5' CCC TCT ACA ACC CAT AAT TTC TGG TCC TCC 3'

Screening primers (to the deletion): hNap1 $\Delta$ 6 $\Delta$ N:

*Top*: 5' GAC CCC AAA GGA ATT CCT GAA TTT TGG 3'

*Bottom*: (pGEX-2T sequencing) 5' CCG GGA GCT GCA TGT GTC AGA GG 3'

Screening primers (to the deletion): hNap1 $\Delta$ 6 $\Delta$ C:

*Top*: (pGEX-2T sequencing) 5' GGG CTG GGA AGC CAC GTT TGG TG 3'

*Bottom*: 5' CTG TCC CAC TGC CCT TGT G 3'

#### GST affinity purification of Nap1 mutants

Purified pGEX-2T plasmid containing human Nap1 (or Nap1 mutant) sequence was transformed into BL21DE3 pLysS cells and incubated overnight at 37°C on LB plates containing ampicillin and chloramphenicol. Cultures were



expanded to 1 L, allowed to grow to  $A_{600} = 0.5$ , and induced using 0.4 mM IPTG. Three hours post induction, cells were harvested using a Beckman J2-21 centrifuge with a JA-10 rotor at 3000 rpm for 30 min. Supernatant was discarded and pellets were resuspended in 10 mL PBS and stored at  $-70^{\circ}\text{C}$  until protein purification.

The pellet was thawed and protease inhibitors were added (1 mM PMSF, 8  $\mu\text{g/mL}$  aprotinin, 8  $\mu\text{g/mL}$  leupeptin, 2 mM DTT). Cells were lysed by sonication using the Branson Sonifier 450 with a flat tip three times for 3 min at output 4/40% with 5 min between each sonication. Tween-20 was added to a final concentration of 1% and the cellular debris was pelleted by centrifugation for 30 min at 14,000 rpm at  $4^{\circ}\text{C}$ . The supernatant was added to equilibrated glutathione agarose resin and incubated with gentle agitation at  $4^{\circ}\text{C}$  for 45 minutes. Resin was separated by centrifugation in a Beckman Allegra 6KR conical centrifuge at 1500 rpm for 10 min at  $4^{\circ}\text{C}$  and the supernatant was removed. Beads were washed three times with PBS+1% Tween-20 by incubating with gentle agitation for 10 min at  $4^{\circ}\text{C}$  followed by centrifugation. Protein was eluted with elution buffer (50 mM Tris pH 7.9, 5  $\mu\text{M}$  reduced glutathione, 2 mM DTT) and dialyzed into 0.1 M TM (50 mM Tris pH 7.9, 100 mM KCl, 12.5 mM  $\text{MgCl}_2$ , 1 mM EDTA, 20% Glycerol) with 2 mM DTT. Concentration was determined using a BSA Protein was stored at  $-70^{\circ}\text{C}$ .

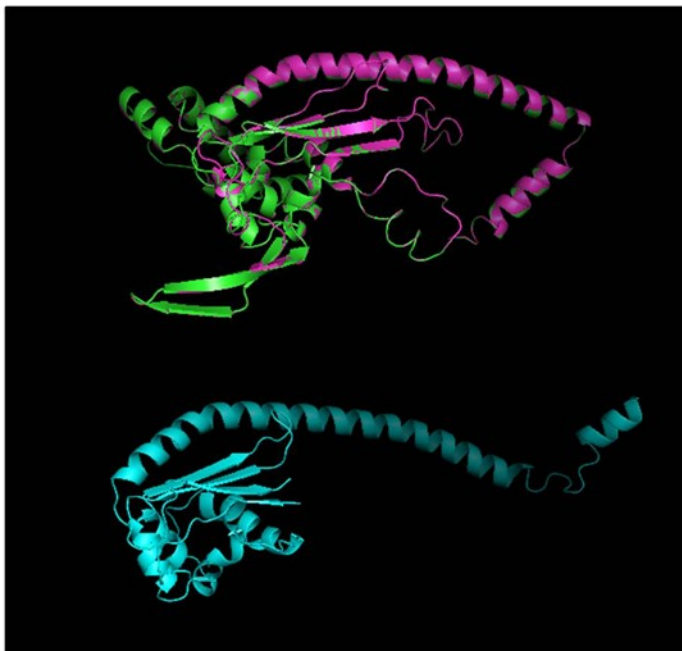
## **2.3 Results**

From the series of truncation mutants our lab previously obtained, we were able to determine that the minimal domain required for histone eviction is

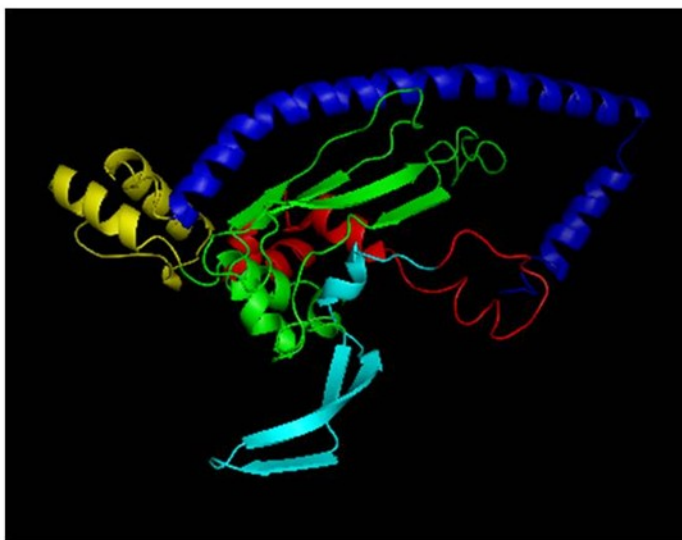
hNap1 $\Delta$ 6, which is comprised of amino acid residues 162-290. Additional deletions of hNap1 $\Delta$ 6 were designed to further test the minimal domain of hNap1 required for nucleosome disassembly at the HTLV-1 promoter. Once constructed, deletion mutants of hNap1 $\Delta$ 6 will supplement data previously obtained in the lab using hNap1 $\Delta$ 1-8 (refer back to figures 8 and 9). The hNap1 $\Delta$ 6 deletion mutants will be tested in GST pull-down and nucleosome disassembly assays, discussed in Chapter 3.

To perform these studies, I designed my deletions in a way that would limit the impact on the structure of the protein. Because human Nap1 has not been crystallized, I used the structural prediction software known as Phyre to aid in the design of the mutants [166, 167]. This program utilizes existing protein structures to predict the structure of the input amino acid sequence, human Nap1. The primary advantage of the Phyre program is that it searches multiple protein databases (e.g. the protein data bank) for crystallized proteins with a high level of sequence homology. Based on the sequence alignments, the program models the structure of the input protein. The use of this program eliminates potential human bias because the user does not provide the structural homologs and the alignment can be performed with multiple proteins to produce multiple models. The output of this program is a structural file along with a map depicting the strength of the sequence alignment between the input protein and the crystallized protein that was used as a model. When provided the sequence of hNap1, the program yielded three viable models based on the structures of yNap and SET/TAF-I $\beta$  (Figure 11).

A



B

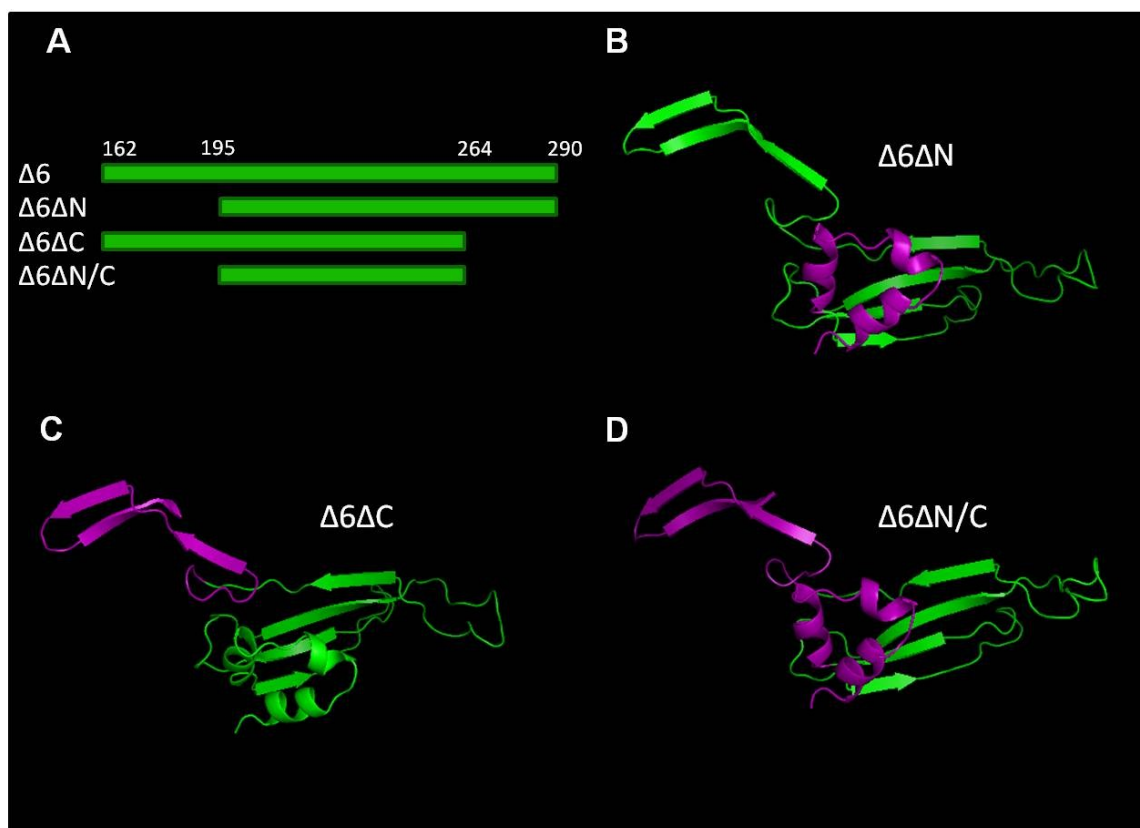


**Figure 11: Predicted structure of human Nap1 can be used to design rational deletion mutants.** (A) Predictions from the Phyre (Protein Homology/Analogy Recognition Engine). Two possible models for human Nap were created based on the yeast NAP crystal structure shown as an overlay in **green** and **magenta**. One structure was generated based on the human SET/TAF-I structure shown in **cyan**. (B) Based on sequence alignment the strongest parts of all three models were used to generate a single model. Colored by domain, the hNap1 $\Delta$ 6 domain is shown in lime green and cyan.

Two models were generated based on the yNap structure, C2AY and D2AY, and one model, C2E5, was generated based on the SET/TAF-I $\beta$  structure (Figure 11A). There are many similarities between the three predicted structures of hNap1. However, areas of divergence occur as a result of parts of yNap and SET/TAF-I $\beta$  were not originally crystallized by the Luger and Horikoshi laboratories [162, 163]. Consequently, there are segments of the hNap1 structure that are not modeled. These sections include some of the flexible linker regions that connect the alpha helices and beta strands.

A single hypothetical crystal structure was generated using the strongest alignments between the sequence of hNap1 and either yNap or SET/TAF-I $\beta$  and the representative portions of their crystal structures. This model was colored to correspond to the different domain coloring of yNap from Park and Luger, 2005 [61] (Figure 11B). This model was used as a platform for the design of deletion mutants that were tested in GST pull-down and nucleosome disassembly assays.

Using Nap1 $\Delta$ 6, colored in green on the combined model (amino acids 162-290), I designed three deletion mutants that should have a limited impact on the structure of the protein (Figure 12A). The first deletion removes the N-terminal alpha helices (amino acids 162-195), leaving amino acids 196-290 (referred to as hNap1 $\Delta$ 6 $\Delta$ N) (Figure 12B). The second deletion removes the C-terminal beta sheet (amino acids 264-290), leaving amino acids 162-263 (referred to as hNap1 $\Delta$ 6 $\Delta$ C) (Figure 12C). The final deletion is a combination of the first and second (removing amino acids 162-195 and 264-290), leaving just 196-293 (referred to as hNap1 $\Delta$ 6 $\Delta$ N/C) (Figure 12D).



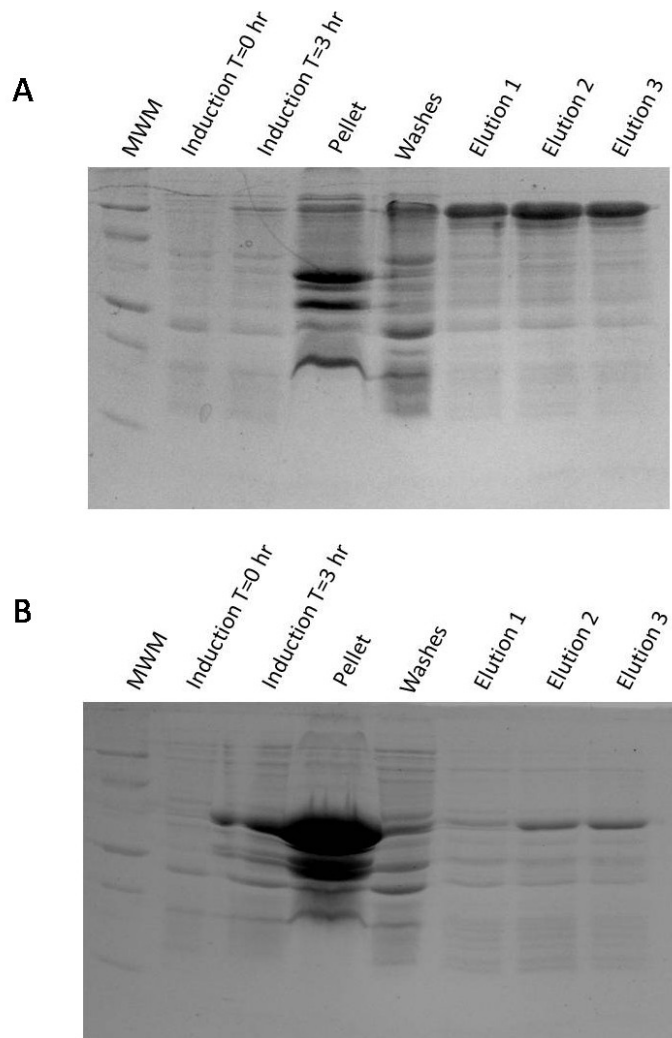
**Figure 12: Rational design of Nap1 deletion mutants.** The full  $\Delta 6$  domain is shown with the deletions in **Magenta** and the remaining part of the protein shown in **Green** (A) Starting with the  $\Delta 6$  domain deletions were made at the N and C terminals and then both terminals were removed. (B) hNap1 $\Delta 6\Delta N$  (C) hNap1 $\Delta 6\Delta C$  (D) hNap1 $\Delta 6\Delta N/C$ .

The rationally designed mutants were generated using a variation of the SLIM cloning technique [164, 165]. This technique utilizes a series of primers to create a deletion using polymerase chain reaction (PCR) for amplification of the plasmid and hybridization of the PCR products. The SLIM method allows for amplification, hybridization, and bacterial transformation in a single day (refer back to Figure 10). Following transformation, colonies were screened using colony PCR with a primer designed to be specific to the deleted sequence. Screening of colonies prior to plasmid purification is critical because theoretically, only half of the colonies contain the correctly hybridized product. Successful deletion was confirmed by the absence of amplification product from the colony PCR reaction. Following plasmid purification, the mutation was also confirmed by DNA sequencing.

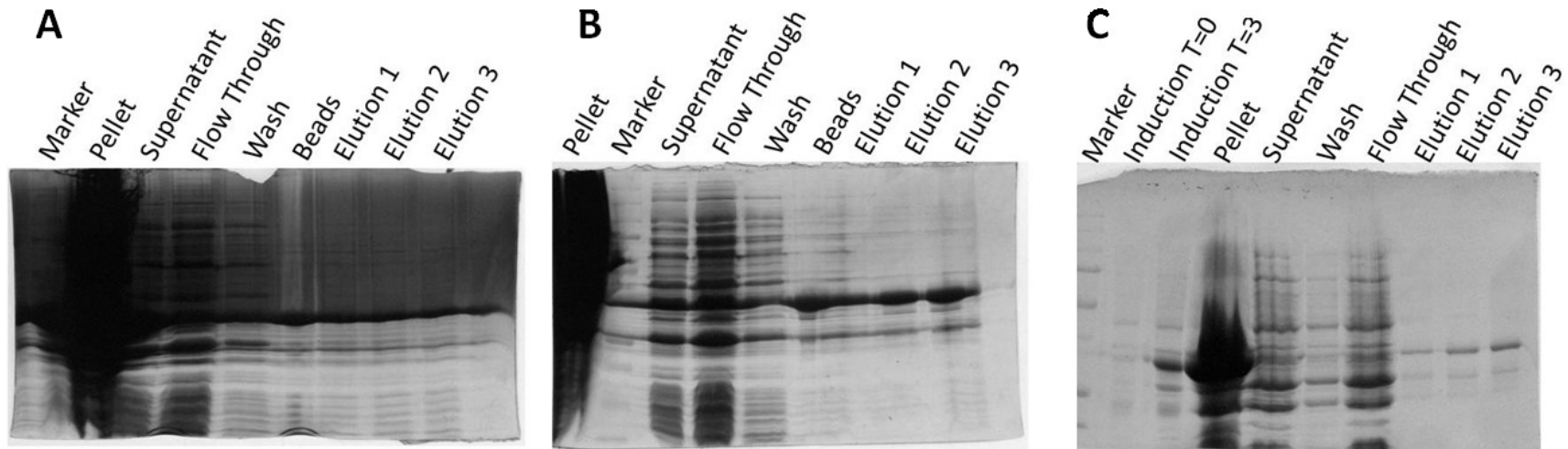
The hNap1 $\Delta$ 6 deletion mutants were purified by GST affinity purification. The purification yielded protein product with similar or greater purity than previous hNap1 $\Delta$ 6 preparations (Figure 13 and 14). Concentration and purity were compared using BSA as a concentration standard on Coomassie stained denaturing polyacrylamide gel (Figure 15).

## **2.4 Discussion**

The structural prediction software known as Phyre was used to generate three models of hNap1 based on the published crystal structures of yeast Nap and human SET/TAF-I $\beta$ . These three models were combined to generate a single model using the strongest sequence alignment to determine the most well predicted portions of the three models. There were regions of all of the models

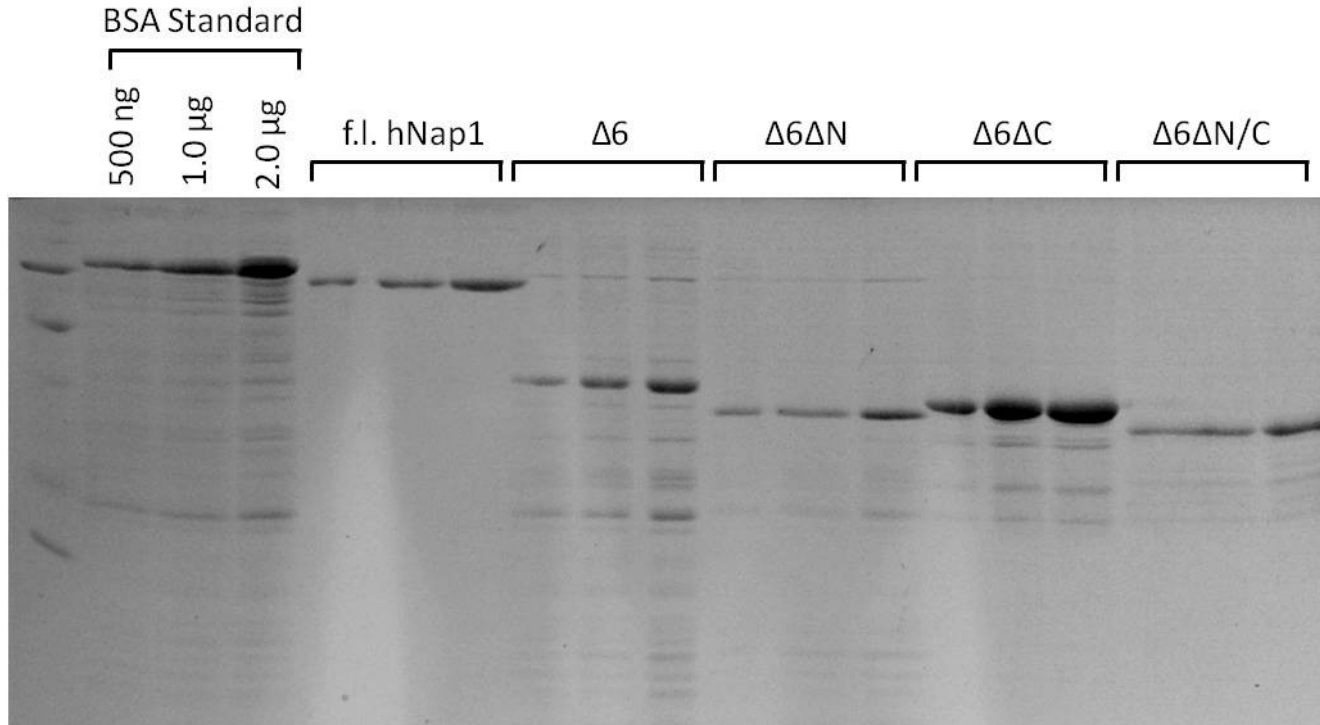


**Figure 13: Full length human Nap1 and  $\Delta 6$  were purified using GST affinity purification.** Proteins were grown using pLysS cells induced with IPTG, harvested and lysed by sonication. After removal of cellular debris, supernatant was bound to glutathione agarose beads, washed, and protein was eluted using reduced glutathione. (A) Purification of Full Length hNap1 (B) Purification of hNap1 $\Delta 6$



**Figure 14: Deletion mutants were purified using GST affinity purification.** Proteins were grown using pLysS cells induced with IPTG, harvested and lysed by sonication. After removal of cellular debris, supernatant was bound to glutathione agarose beads, washed, and protein was eluted using reduced glutathione. (A) Purification of hNap1 $\Delta$ 6 $\Delta$ N (B) Purification of hNap1 $\Delta$ 6 $\Delta$ C (C) Purification of hNap1 $\Delta$ 6 $\Delta$ N/C





**Figure 15: Comparison of yield and purity of full length Nap1,  $\Delta 6$ ,  $\Delta 6\Delta N$ ,  $\Delta 6\Delta C$ ,  $\Delta 6\Delta N/C$ .** BSA was used as a standard for determining the concentration of the purified proteins. Post-dialysis sample was loaded onto a 13% denaturing polyacrylamide gel with SDS sample buffer. Titrating proteins: 1, 2, and 4 microliters of each protein post dialysis for comparison on purity and relative concentration.

that lacked predicted structure. This is likely a result of unstructured regions of the protein or portions of the proteins that exist in multiple conformations. These regions are not shown in the hNap1 model crystal structure. From these structures, I determined the most rational locations to design deletion mutations of hNap1 $\Delta$ 6.

Although the alignment was very strong, it must be mentioned that the generated structure is only a prediction. The actual human Nap1 structure may vary slightly in terms of what residues are in  $\alpha$ -helices and  $\beta$ -sheets or in flexible linker regions, etc. It is also possible, however unlikely, that the predicted structure is an inaccurate representation of the actual hNap1 structure.

The hNap1 $\Delta$ 6 deletion mutants were cloned using a variation of the published SLIM technique. The creation of the deletions was confirmed by sequencing. Following bacterial expression, purification of the hNap1 deletion mutants produced lower protein yields than previous preparations of GST tagged full length hNap1. Full length GST-hNap1 is cloned into the same plasmid and was expressed under the same conditions. The difference in yields could be a result of precipitation of the protein due interaction of the hydrophobic patches that were potentially exposed in the design of the hNap1 deletion mutants. Adequate amounts of protein was obtained to continue with the functional studies, however optimization of the expression and purification process is discussed in the future directions section of chapter 4.

**CHAPTER 3**

**FUNCTIONAL CHARACTERIZATION OF HUMAN NAP1 DELETION  
MUTANTS AND THEIR FUNCTION IN NUCLEOSOME DISASSEMBLY AT  
THE HTLV-1 PROMOTER**

**3.1 Background**

This chapter will focus on the function of human Nap1 and will utilize the mutants designed in the previous chapter for in vitro analysis in histone binding and nucleosome disassembly assays. Previous reports from our laboratory described an immobilized template assay utilizing the HTLV-1 promoter as a DNA template [60]. It has been established that nucleosome disassembly and transcription activation at the HTLV-1 promoter requires recruitment of the Ser133 phosphorylated form of the cellular protein, CREB (pCREB), together with the viral protein, Tax. Phosphorylated CREB forms a homodimer that interacts with the central region of the viral CRE. In addition, two Tax molecules make protein-protein interactions with pCREB while simultaneously interacting with the GC-rich regions of the DNA in the vCRE. Together, Tax and pCREB recruit the cellular coactivator and histone acetyltransferase, p300. Tax and pCREB bind the KIX domain of p300. The protein-DNA complex formed with Tax, pCREB, p300 and the vCRE is very stable. Upon recruitment, p300 acetylates the histone tails of promoter nucleosomes.

In addition to formation of the quaternary complex, the presence of the histone chaperone, Nap1, is also required for nucleosome disassembly from the HTLV-1 promoter. In our assay, multiple proteins in the complex at the HTLV-1 promoter may interact with Nap1. Nap1 may be recruited to the promoter by p300 because Nap1 and p300 are shown to interact in vitro [59, 148, 149]. Unpublished data from the Nyborg laboratory has also shown by GST pull-down and coimmunoprecipitation that Nap1 and Tax interact.

As discussed in Chapter 2, the minimal domain of hNap1 functional in nucleosome disassembly assays is hNap1 $\Delta$ 6, which encompasses residues 162-290. The research discussed in this chapter aims to utilize additional deletion mutants (as designed in the previous chapter) to provide a foundation for future studies to identify the specific residues of Nap1 required for Nap1-dependent nucleosome disassembly.

### **3.2 Materials and Methods**

#### **CREB expression, phosphorylation, and purification**

CREB was purified as outlined in [168].

CREB was cloned into pET-3B, an inducible *E. coli* expression plasmid [106]. PET-3b-CREB was transformed into Rosetta BL21DE3 pLysS competent cells and incubated overnight at 37°C on LB agar plates containing ampicillin and chloramphenicol. Colonies were expanded to 1 L, grown to an OD<sub>600</sub> of 0.5-0.8, and induced using 0.4 mM IPTG. Three hours post induction, cells were pelleted in a Beckman J2-21 centrifuge with a JA-10 rotor at 3,000 rpm for 30 min at 4°C. Supernatants were discarded and pellets were resuspend in 15 mL CREB

Resuspension Buffer per liter of culture (50 mM Tris pH 7.9, 12.5 mM MgCl<sub>2</sub>, 100 mM KCl, 1 mM EDTA pH 8.0, 10 µM ZnSO<sub>4</sub>) and stored at -70°C.

The pellet was thawed, incubated 15 min at 65°C, and placed back on ice. Protease inhibitors, RNase A, and reducing reagent were added to the resuspended pellet (1 mM PMSF, 8 µg/mL aprotinin, 8 µg/mL leupeptin, 2 mM DTT, and 200 µg/mL RNase A). Resuspended pellet was centrifuged at 14,000 rpm for 30 min at 4°C. Supernatants were combined and the concentration was determined by Bradford assay.

PKA phosphorylation of CREB was carried out at 30°C for 30 min in PKA reaction buffer (20 mM Tris pH 7.5, 1 mM NaF, 1 mM MgCl<sub>2</sub>), with 3000 Units PKA, and 20 µM ATP and 8 mM DTT. The final concentration of CREB in the reaction was 1 pmol/µL.

Phosphorylated CREB was incubated with gentle agitation on heparin sepharose resin overnight at 4°C. Protein-bound resin was transferred to a Bio-Rad column and washed with CREB Resuspension buffer (50 mM Tris pH 7.9, 12.5 mM MgCl<sub>2</sub>, 100 mM KCl, 1 mM EDTA pH 8.0, and 10 µM ZnSO<sub>4</sub>, 2 mM DTT, 1 mM PMSF). Phosphorylated CREB was eluted from the column using 8 M Urea Buffer (8 M Urea, 1 M NaCl, 50 mM Na<sub>2</sub>PO<sub>4</sub> pH 8.0, 2 mM DTT, 1 mM PMSF) and concentrated using an Amicon Ultra-15 (MWCO 10K) conical to a final volume of 2 mL from an initial elution volume of 60 mL. Concentrated protein was centrifuged at 14,000 rpm for ten minutes to eliminate precipitates from the final product.

A HiLoad Superdex 200 16/60 column (Amersham Cat # 17-1069-10) was equilibrated in 8 M Urea Buffer before the addition of pCREB. Concentrated pCREB was loaded onto the Superdex column and eluted in 1.5 mL fractions. Purified pCREB was dialyzed at room temperature in CREB Dialysis Buffer (50 mM Na<sub>2</sub>PO<sub>4</sub>, 100 mM NaCl, 1 mM MgCl<sub>2</sub>, and 2 mM DTT) before storage at -70°C.

#### Tax Expression and Purification

The pTaxH6 plasmid for His<sub>6</sub>-tagged Tax [123] was transformed into competent pLysS cells and incubated overnight at 37°C on LB agar plates containing ampicillin and chloramphenicol. Cultures were expanded to 2 liters and cultures grown for 13 hours at 37°C. Cells were pelleted in a Beckman GPKR at 3600 rpm for 30 min at 4°C and resuspended in 40 mL Tax Lysis Buffer (100 mM Tris pH8, 100 mM KCl, 10 µM ZnSO<sub>4</sub>) and stored at -70°C until purification.

The pellet was thawed and 50 mM imidazole and protease inhibitors were added (1 mM PMSF, 8 ng/µL leupeptin, 8 ng/µL aprotinin, 4 mM β-Me, and 1 EDTA free protease inhibitor tablet Roche Product #11 873 580 001). Resuspended cells were lysed by sonication using a Branson Sonifier 450 with a flat tip at three times for 3 min at output 6/40% with 5 min rest between each sonication. Cellular debris was pelleted by centrifugation in Oakridge tubes in a Beckman J2-21 centrifuge with a JA-20 rotor at 15,000 rpm for 30 min at 4°C.

Supernatant was bound to equilibrated Ni-NTA slurry by incubating with gentle agitation at 4°C overnight. Resin was transferred into a column, washed,

and eluted in 1 mL fractions with a 0 mM to 300 mM imidazole gradient. Peak protein fractions were pooled and concentration was determined by Coomassie stained 12% SDS-page. BSA was used as a concentration standard. Following purification, protein was stored at -70°C.

#### *p300 Expression and Purification*

His<sub>6</sub>-tagged p300 was expressed from recombinant baculovirus in Sf9 cells cultured in spinner flasks [169]. A 1 L culture at  $1 \times 10^6$  cells/mL was infected with 100 mL of recombinant baculovirus. Cells were harvested 60 hours post infection when they showed slight enlargement and a granular appearance. Cells were pelleted by centrifugation and flash frozen in liquid nitrogen and stored at -70°C.

The pellet was thawed and resuspended in homogenization buffer (10 mM Tris pH 7.5, 500 mM NaCl, 10% Glycerol, 0.1% NP-40, 15 mM Imidazole, 2 mM  $\beta$ -Me, 2 mM PMSF, 20  $\mu$ g/mL leupeptin, and 20  $\mu$ g/mL aprotinin) and homogenized on ice using a Dounce homogenizer, tight pestle. After centrifugation 30 min/4°C at 2000 rpm in a Beckman Allegra 6KR Centrifuge, the supernatant was incubated with gentle agitation for 2 hr at 4°C with equilibrated Ni-NTA slurry. Beads were pelleted at 2000 rpm and washed 3 times with wash buffer (10 mM Tris pH 7.5, 200 mM NaCl, 10% Glycerol, 0.2% NP-40, 15 mM Imidazole, 2 mM  $\beta$ -Me, 2 mM PMSF). Protein was eluted using elution buffer (10 mM Tris pH 7.5, 10 mM NaCl, 10% Glycerol, 0.1% NP-40, 500 mM Imidazole, 2 mM  $\beta$ -Me, 2 mM PMSF) and dialyzed into 0.1 M TM (50 mM Tris pH 7.9, 100 mM KCl, 12.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 20% Glycerol) with 2 mM DTT.

Acetyltransferase activity was tested using radiolabeled acetyl CoA and histone substrates. The purified p300 was stored at -70°C.

#### Purification of hNap1 and the Nap1 Deletion Mutants

Refer to Chapter 2 for cloning, expression and purification of hNap1 and the deletion mutants.

#### -306 fragment amplification and purification

A 588 bp fragment carrying the full HTLV-1 promoter upstream of a G-less cassette was amplified and biotinylated by PCR (referred to as -306, the position at which the fragment begins relative to the transcription start site at +1).

Top Primer Sequence:

5' Bio/5' GTC TGA AAA GGT CAG GGC C 3'

Bottom Primer Sequence:

5' GGA TAT ATG AGA TGA GTA GG 3'

PCR reactions contained 500 ng of template DNA, 200 ng each primer, Polymerase Reaction Buffer to a final concentration of 1X, 200 nmol dNTP mixture, and 25 units Taq polymerase to produce 1 mL of final PCR product. The fragment was amplified using the following protocol: 2 min at 94°C then allowed to proceed for 40 cycles (94°C for 30 sec, 45.5°C for 1 min, 72°C for 45 sec) followed by an additional elongation step at 72°C for 5 min and a hold step at 4°C.

Phenol/chloroform/isoamyl alcohol (IAA) extraction was used to purify biotinylated -306. Equal volume of phenol/chloroform/IAA was added followed by vortexing and centrifugation in a Beckman J2-21 centrifuge with a JA-20 rotor at



5000 rpm for 5 min. The top phase was removed for DNA purification and the bottom and interphases were discarded.  $\text{NH}_4\text{OAc}$  was added to a final concentration of 2 M and 2.5X the volume of cold 100% EtOH was added. The mixture was vortexed and placed in the  $-70^\circ\text{C}$  freezer overnight to aid in precipitation followed centrifugation at 5000 rpm for 30 min at  $4^\circ\text{C}$ . Supernatant was discarded and pellets were washed with cold 70% EtOH. DNA was pelleted by centrifugation at 5000 rpm for 30 min at  $4^\circ\text{C}$ . Supernatant was removed and the pellet was allowed to air dry. Purified biotinylated 588 bp DNA fragment was resuspended in  $\text{H}_2\text{O}$ .

#### *Immobilized Template Chromatin Assembly*

Biotinylated -306 HTLV-1 promoter fragment was incubated with Streptavidin coupled magnetic Dynabeads® (Invitrogen catalog number 112-05D) for 1 hour while shaking at  $30^\circ\text{C}/1200$  rpm in an eppendorf Thermomixer R. After binding, -306 bound to Dynabeads was stored in 10 mM Tris, 1 mM EDTA (1XTE) or assembled into chromatin.

Chromatin was assembled using the salt dilution method. An empirically determined optimal core histone to DNA ratio was incubated in 1XTE for 40 min at  $4^\circ\text{C}/1200$  rpm with a starting NaCl concentration of 1 M. At 40 minute intervals, 1XTE was used to dilute the reaction to NaCl concentrations of 0.9 M, 0.8 M, 0.6 M, 0.4 M, 0.2 M, and 0.1M respectively. Sample was magnetically separated and the supernatant was removed. Chromatin bound to the magnetic beads was resuspended in storage buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA, 5

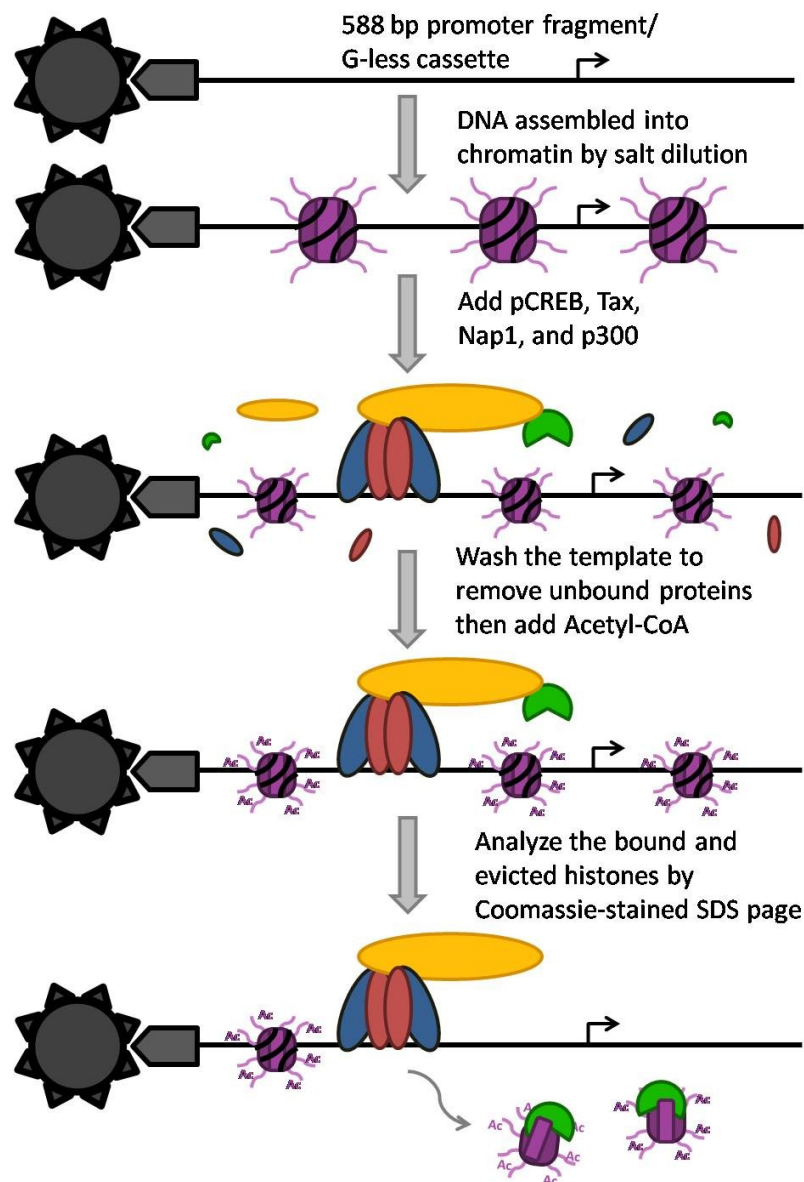
mM DTT, 0.1% NP-40, 20% Glycerol, 100 mM NaCl) to a final DNA concentration of 100 ng/μL.

#### Micrococcal Nuclease Assay

Micrococcal nuclease (MNase) assay was used to analyze for correctly assembled chromatin. Chromatin (0.6 μg for each desired time point) was resuspended in micrococcal Buffer (20 mM Tris HCl pH 8.0, 5 mM NaCl, 2.5 mM CaCl<sub>2</sub>) and incubated 5 min at 37°C/1200 rpm. Prior to treatment with MNase, the t=0 time point was removed and the remaining chromatin was treated with 1 unit of MNase per remaining time point. Time points were determined arbitrarily to capture the digested chromatin when it exists as a mixture of trinucleosome, dinucleosome, and mononucleosome. The digested sample was removed and added to MNase stop buffer (5 mM Tris, 250.5 mM EDTA). Proteinase K buffer (250 mM NaCl, 1% SDS, 20 mM Tris HCl pH 7.5, 5 mM EDTA) was added to the reaction in a 4/3 ratio to the volume of the stopped reaction with 50 μg of Proteinase K and incubated for 30 min at 30°C/1200 rpm. DNA was extracted using phenol/chloroform/isoamyl alcohol and samples were separated on a 1.5% agarose-TBE gel. DNA fragment was visualized using ethidium bromide.

#### Eviction Assay

Refer to Figure 16 for a schematic of the eviction assay. The biotinylated HTLV-1 promoter bound to a magnetic streptavidin bead was assembled into chromatin using salt deposition. For each reaction, 1 μg assembled chromatin was resuspended in 0.1 M Eviction TM (50 mM Tris pH 7.9, 100 mM KCl, 6.25 MgCl<sub>2</sub>, 1 mM EDTA, 20% glycerol) containing 2 mM DTT. Chromatin was



**Figure 16: Schematic showing the nucleosome disassembly assay.** The disassembly assay begins with assembly of chromatin on the biotinylated 588 bp promoter fragment of HTLV-1 bound to a magnetic streptavidin bead. Chromatin is assembled by salt dilution method. Activators pCREB and Tax are incubated with assembled chromatin followed by addition of the coactivator, p300 and the histone chaperone human Nap1. Following assembly, the template is washed to remove excess proteins and Acetyl CoA is added to complete the disassembly assay. Acetylated histones are removed from the template in a Nap1 and Acetyl CoA dependent manner. The exact mechanism for disassembly is currently unknown. [Key: pCREB = Red, Tax = Blue, p300 = Yellow, hNap1 = Green, histones = Purple]

incubated for 20 min at 30°C/1200 rpm with 60 pmol of both pCREB and Tax. The acetyltransferase, p300, was added based on relative HAT activity with 45 pmol human Nap1 (or mutants of human Nap1) and incubated for 30 min at 30°C/1200 rpm. Following incubation, the unbound fraction may be removed for clarified eviction results or may remain in the sample to prevent shifting of the equilibrium of Tax/pCREB/p300 complex formation. Acetyl CoA was added (<sup>14</sup>C radiolabeled acetyl CoA can also be used to monitor acetylation patterns of the four core histones and other proteins in complex at the HTLV-1 promoter). The bead-bound fraction was incubated for 40 min at 30°C/1200 rpm. The bound and evicted fractions were mechanically separated by magnetic isolation and the bead-bound fragment was washed with 0.1M TM buffer. Samples were resuspended in SDS loading dye and separated on a 13% denaturing polyacrylamide gel. Histones were visualized by Coomassie staining.

#### GST Pull-down Assay

GST-tagged Nap1 mutants were used to investigate the domain of Nap1 required for Nap-histone interactions. Positive controls for Nap1-histone interactions included full length Nap1 and Nap1Δ6 with histones. As a positive control for the GST pull-down assay, the GST-KIX domain of p300 was tested for interaction with pCREB (the pCREB and KIX interaction has been well characterized in our laboratory). GST protein alone was used as a negative control for histone binding. Glutathione agarose beads were incubated with 20 pmol of the GST fusion proteins for 2 hours with gentle agitation at 4°C in 0.5XSuperdex-150 buffer (12.5 mM HEPES pH 7.9, 6.25 mM MgCl<sub>2</sub>, 5 μM

ZnSO<sub>4</sub>, 150 mM KCl, 10% glycerol, and 0.5% Tween-20). Samples were washed three times with 0.5XSuperdex-150. Approximately 10 pmol of the secondary protein (either octamer with GST-Nap1 proteins or pCREB with GST-KIX) was incubated with gentle agitation at 4°C overnight. Samples were washed three times with 0.5XSuperdex-150 buffer to reduce non-specific interactions, separated on a 13% denaturing acrylamide gel, and visualized by Coomassie staining.

### **3.3 Results**

Functionality of the three new hNap1 deletion mutants that were designed, cloned, expressed, and purified (as described in chapter 2) were tested in a combination of in vitro assays. Specifically, the histone binding properties of mutants were assayed for histone binding by GST pull-down assay and their ability to support nucleosome disassembly using the immobilized template assay.

#### ***3.3a Analysis of hNap1-histone interaction by GST pull-down assay***

We hypothesized that if a hNap1 mutant is unable to interact with histones, then that hNap1 mutant protein would be unable to support disassembly of nucleosomes from the HTLV-1 promoter. GST pull-down assays allow for characterization of protein-protein interactions based on specific binding as well as non-specific electrostatic interactions. Briefly, each GST-hNap1 protein was incubated with increasing concentrations of purified recombinant *Xenopus* histones. GST protein was used as a negative control for histone interaction. Full length GST-hNap1 and GST-hNap1Δ6 were used as positive

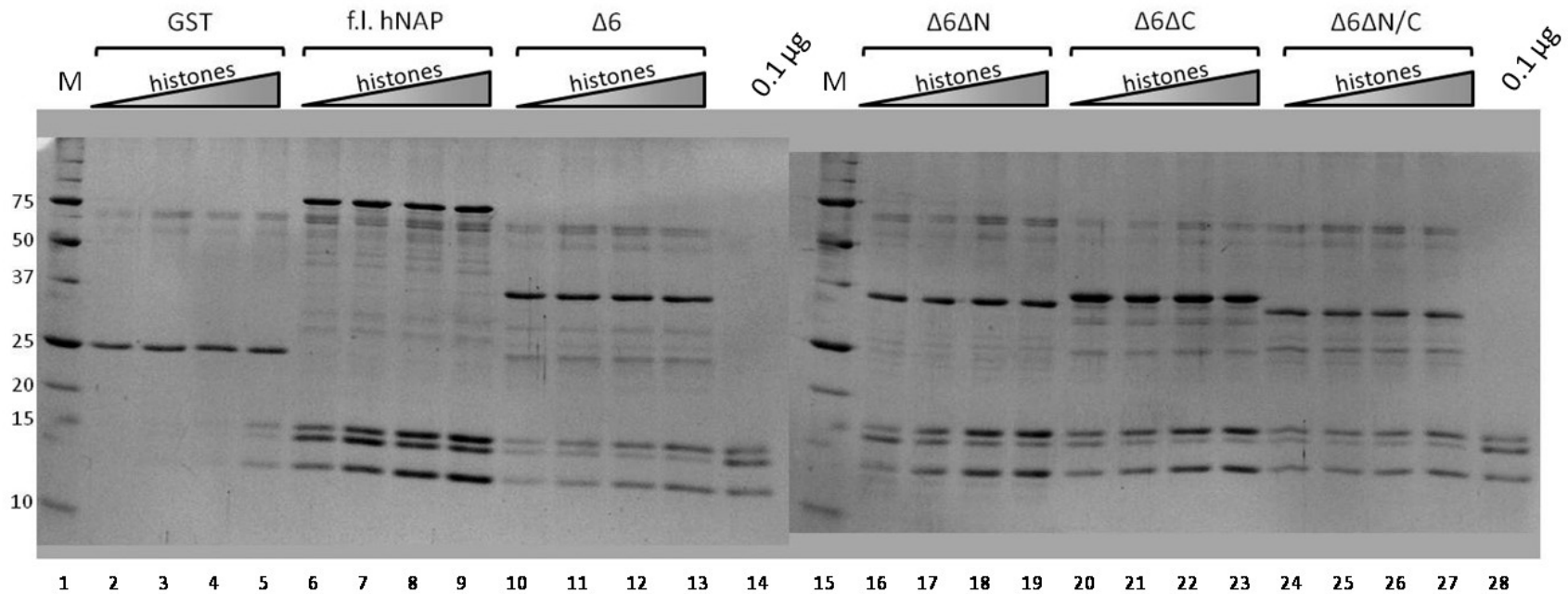
controls because both proteins bind histones and support nucleosome disassembly (see Figure 17).

GST protein alone was used to monitor non-specific interactions with GST because all of the hNap1 constructs are GST-tagged. GST has non-specific interactions with histones only at the highest concentration of octamer (Figure 17, lanes 2-5).

Full length hNap1 appears to bind all four core histones with equal affinity (H2A, H2B, H3, and H4) (Figure 17, lanes 6-9) and reaches histone binding saturation upon incubation with 1  $\mu$ g of histone octamer (Figure 17, lane 7).

GST-hNap1 $\Delta$ 6 was used for the GST pull-down assay because previous work in our laboratory demonstrated that hNap1 $\Delta$ 6 is the minimal domain of hNap1 able to support histone eviction. In the GST pull-down assay (Figure 17), at low concentrations of histone octamer, hNap1 $\Delta$ 6 binds all four core histones with equal affinity (Figure 17, lanes 10 and 11). As the concentration of octamer is increased, hNap1 $\Delta$ 6 demonstrates a clear preference of H3/H4 binding (Figure 17, lanes 12 and 13).

The two hNap1 $\Delta$ 6 deletion mutants, hNap1 $\Delta$ 6 $\Delta$ N and hNap1 $\Delta$ 6 $\Delta$ C both appear to possess a higher affinity for all four core histones than the parent, hNap1 $\Delta$ 6, at all concentrations of histone octamer (Figure 17, lanes 16-23). Binding of histones H3/H4 to the hNap1 mutants increased as the concentration of octamer in the reaction increase, while H2A/H2B remained constant despite the increasing amount of octamer present (Figure 17, lanes 19 and 23). The third mutant, hNap1 $\Delta$ 6 $\Delta$ N/C has a decreased affinity for the both H2A/H2B and



**Figure 17: GST pull-down using deletion mutants assayed for binding of increasing amounts of *Xenopus* wild type histone octamer.** Each sample contains 20 pmol of hNap1 or hNap1 mutant. Histone octamer is titrated in increasing amounts. Human Nap1 bound to GST agarose beads was incubated with either 0.5  $\mu$ g, 1  $\mu$ g, 2  $\mu$ g, or 4  $\mu$ g of *Xenopus* wild type octamer. A 0.1  $\mu$ g input is shown in lanes 14 and 28. This is equivalent to 10% of 1.0  $\mu$ g, the amount of histones in the reactions shown in lanes 3, 7, 11, 17, 21, and 25. Marker is shown in lanes 1 and 15.

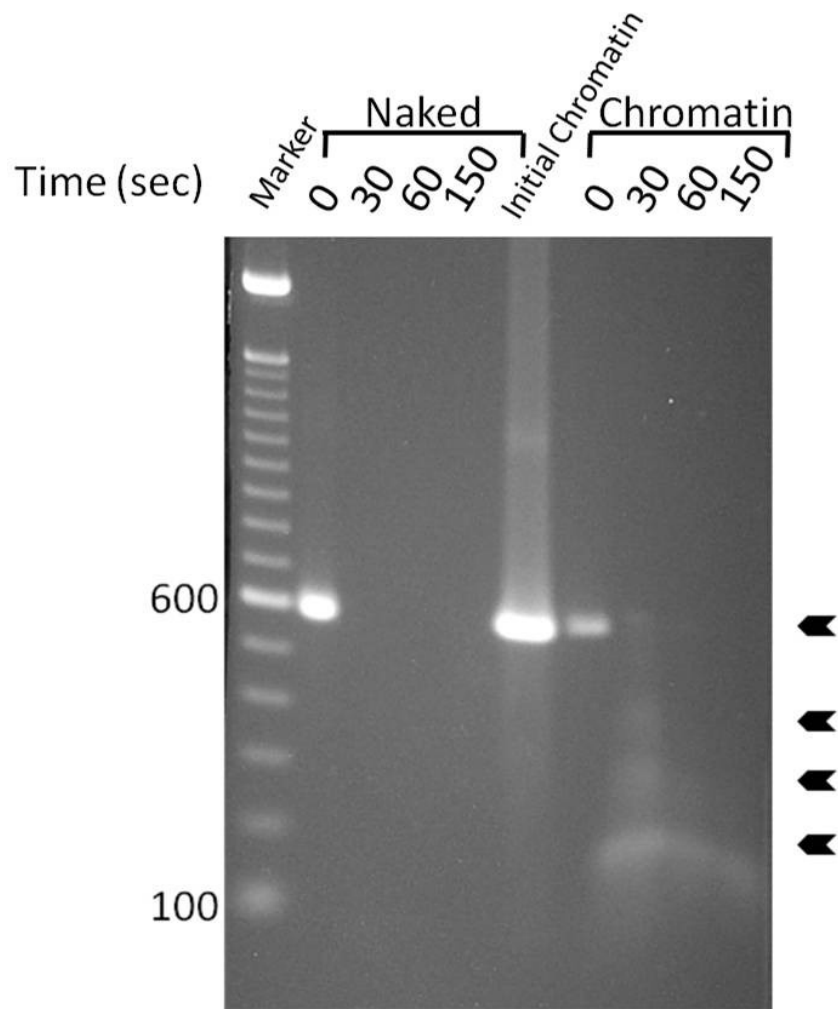
H3/H4 compared to the other two mutants. Overall, the histone binding pattern of hNap1 $\Delta$ 6 $\Delta$ N/C is similar to hNap1 $\Delta$ 6, maintaining a higher affinity for H3/H4 compared to H2A/H2B.

### ***3.3b Analysis of the Nucleosome Disassembly Activity of the hNap1 Deletion Mutants***

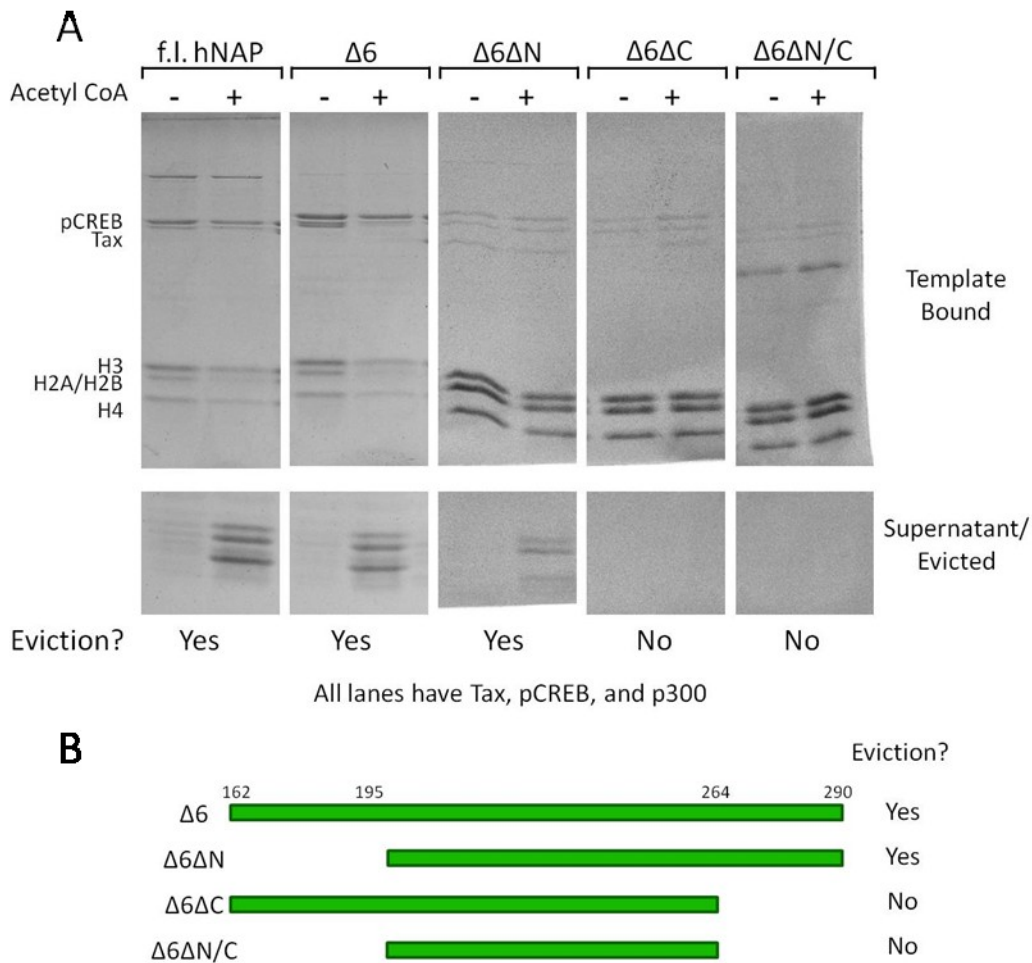
We hypothesized that if a deletion mutant is unable to disassemble nucleosomes from the HTLV-1 promoter, then this portion of hNap1 performs a critical function in the disassembly reaction. To test the ability of the hNap1 deletion mutants to support disassembly, the immobilized HTLV-1 -306 promoter template was assembled into chromatin using the salt deposition method. The integrity of the chromatin was examined by micrococcal nuclease assays (Figure 18). As described in figure 16, the template was incubated with Tax and pCREB followed by addition of p300 and hNap1. Finally, acetyl CoA and acceptor DNA are added, and the bound and evicted fractions are separated by magnetic isolation. Following isolation the samples are separated using SDS-PAGE and visualized using Coomassie staining (Figure 19). The appearance of evicted histones in the supernatant correlates with a decrease in histones remaining bound to the template.

Full length hNap1 and hNap1 $\Delta$ 6 were used as positive controls for the disassembly assay. A disassembly assay was performed on both hNap1 and hNap1 $\Delta$ 6 in the presence and absence of acetyl CoA. In the presence of Tax, pCREB, p300, acetyl CoA, acceptor DNA and Nap1 (or Nap1 $\Delta$ 6) nucleosomes





**Figure 18: Micrococcal nuclease assay reveals approximately three nucleosomes assembled onto the HTLV-1 promoter.** Micrococcal nuclease digests the DNA that is not protected by histone proteins. Unassembled DNA is used as a control to determine the effectiveness of the nuclease. At all time points, the naked DNA is completely digested. The recovery standard is used to visualize the amount of DNA at the beginning of the reaction prior to digestion and DNA extraction. The chromatin is digested with nuclease for 30 seconds, 1 minute, and 2 minutes. At 30 seconds, undigested template, trinucleosome, dinucleosome, and mononucleosome are indicated with black arrows. As digestion progresses at  $t=1:00$  and  $t=2:30$ , the final product is the DNA protected by a single nucleosome, approximately 150 base pairs.



**Figure 19: Deletions lacking the C-terminus of the  $\Delta 6$  domain of human Nap1 are unable to evict nucleosomes from the HTLV-1 promoter template.** (A) Eviction assays were performed using the Nap1 deletion mutants as outlined in figure 17. All samples contain Tax, pCREB, and p300 along with one of the Nap1 mutants. Full length Nap1 and the  $\Delta 6$  domain were used as positive controls, samples lacking acetyl CoA were used as negative controls. (B) Linear model of the  $\Delta 6$  deletion mutants used in eviction assays with a summary of the eviction results.

are disassembled. Binding of histones to the template is noticeably diminished concurrent with appearance of histones in the supernatant.

The eviction assay was also performed on the hNap1 deletion mutants designed in chapter 2. Of the three new hNap1 $\Delta$ 6 deletion mutants, hNap1 $\Delta$ 6 $\Delta$ N is the only mutant able to disassemble nucleosomes from the HTLV-1 promoter. Overall, deletions lacking the C-terminus of hNap1 $\Delta$ 6 domain, including hNap1 $\Delta$ 6 $\Delta$ C and hNap1 $\Delta$ 6 $\Delta$ N/C, were defective for disassembly function (Figure 19). The deletion that removes the C-terminus of hNap1 $\Delta$ 6 contains the  $\beta$ -hairpin from amino acids 264-290, which is involved in Nap1 oligomerization [170].

### **3.4 Discussion**

Previous reports have shown that the  $\beta$ -hairpin of Nap1 is not involved in histone interaction (Andrews and Luger unpublished, refer to [170]). GST pull-downs with Nap1 mutants lacking this  $\beta$ -hairpin do not show distinctly different histone binding patterns compared to hNap1 $\Delta$ 6 mutants containing the  $\beta$ -hairpin, including hNap1 $\Delta$ 6 and hNap1 $\Delta$ 6 $\Delta$ N. The reduced binding affinity of Nap1 $\Delta$ 6 and the other mutants to H2A/H2B could be a result of deletion of the C-terminal acidic domain of hNap1, which associates with H2A/H2B [74]. This domain is not present in Nap1 $\Delta$ 6 or any of the deletion mutants we designed using hNap1 $\Delta$ 6 as a parent, including Nap1 $\Delta$ 6 $\Delta$ N, Nap1 $\Delta$ 6 $\Delta$ C, and Nap1 $\Delta$ 6 $\Delta$ N/C.

Interestingly, there is contradictory evidence regarding the affinity of Nap1 for dimer compared to tetramer. Our GST pull-down histone binding studies elicit a difference in affinity for dimer and tetramer, with an apparent diminished

capacity to bind H2A/H2B. It has been demonstrated that under physiological assay conditions used in the pull-down assays histone octamer dissociates to dimer and tetramer [171]. In vivo studies show that Nap1 coimmunoprecipitates with H2A/H2B, but not H3/H4 [172, 173]. However, in vitro, Nap binds to all four core histones [71, 73, 174-176]. Some studies have shown that Nap1 has a preference for H2A/H2B [173], but in vitro pull-down assays performed in the Luger laboratory demonstrated that when incubated with either dimer or tetramer, the affinities were approximately equivalent, but when incubated with octamer (which dissociates to dimer and tetramer), Nap has a greater affinity for tetramer [73]. Further interaction based studies with separate dimer and tetramer may provide more information about mutant Nap1-histone affinities. Binding studies performed with GST-hNap1 mutants and histone octamer do not show a correlation for histone binding capacity and nucleosome disassembly function.

Because the C-terminal deletions do not evict, the C-terminus would be an ideal location to begin making point mutations. It is interesting to note that this  $\beta$ -hairpin is involved in oligomerization [170], and that in the conditions of the nucleus, Nap exists as a mix of dimer and octamer [177]. This suggests a direct role for the oligomeric form of hNap1 in disassembly of nucleosomes from the HTLV-1 promoter. Oligomerization of Nap1 occurs through the formation of a  $\beta$ -sheet from the  $\beta$ -hairpins of two separate Nap1 monomers. The formation of a  $\beta$ -sheet occurs through the interaction of nine hydrogen-bonding sites between the  $\beta$ -hairpins [170]. When the  $\beta$ -hairpin is deleted in Nap1 $\Delta$ 6 $\Delta$ C and Nap1 $\Delta$ 6 $\Delta$ N/C, these mutants would not be able to oligomerize.

In addition to the disruption of the oligomerization of hNap1, there are multiple reasons one of the mutants may be unable to support disassembly of nucleosomes. It is possible that removing this portion of the protein causes a structural disturbance and the protein becomes misfolded. It would be beneficial to design a series of point mutations that would disrupt the hydrogen bonding of the  $\beta$ -hairpins, and thus oligomer formation, and test these mutants in disassembly reactions.

In conclusion, it appears that the oligomerization of Nap1 is critical for nucleosome disassembly from the HTLV-1 promoter. Further studies with full length Nap1 are required to confirm this result (discussed in the Future Directions section of Chapter 4).

## CHAPTER 4

### FUTURE DIRECTIONS

In the preceding chapters we explored the different domains of Nap1 and their function in histone binding and nucleosome disassembly assays. Previous unpublished work from the Nyborg laboratory was used to identify amino acid residues 162-290 as the minimal domain of Nap1 required for the disassembly of nucleosomes. This domain is referred to as Nap1 $\Delta$ 6 and was the starting point for making further deletion mutants. In addition, we predicted a crystal structure of human Nap1. This predicted structure aided us in determining rational locations to begin making deletion mutants.

Briefly, Nap1 deletion mutants were cloned using a novel technique known as SLIM and subsequently expressed in and purified from *E. coli*. Nap1 deletion mutants were then tested in functional assays, including GST pull-downs and immobilized template assays. From these studies, we determined that Nap1 $\Delta$ 6 $\Delta$ C and Nap1 $\Delta$ 6 $\Delta$ N/C are unable to disassemble nucleosomes from the HTLV-1 promoter. Of note, both of these mutants lack the C-terminal residues of Nap1 $\Delta$ 6, referred to as the  $\beta$ -hairpin domain, which is involved in Nap1 oligomerization, suggesting that Nap1 oligomerization is necessary for nucleosome disassembly from the HTLV-1 promoter.

We propose these future studies to further characterize the functional state of Nap1 required for nucleosome disassembly from the HTLV-1 promoter template.

#### **4.1 Optimization of Nap1 Purification**

In consideration of possible confounding variables, optimizing the expression and purification of the three Nap1 deletion mutants, as well as the  $\Delta 6$  domain, could provide more conclusive results.

Although protein expression was successful and protein was obtained to perform the studies, after sonication much of the protein remained in the cell pellet, resulting in less than optimal purification yields. Additional purification steps could be added to purify the proteins, such as:

1. The Nap1 mutant proteins may be insoluble due to hydrophobic patches becoming exposed in the process of deleting domains of Nap1. The presence of Nap1 mutants in the cell pellet suggests that the insoluble proteins are forming inclusion bodies. An inclusion body preparation would extract the insoluble protein and denaturation would allow for proper refolding of the proteins.
2. A second GST purification step may separate Nap1 from other proteins that interact non-specifically with the glutathione agarose beads or with Nap1 itself.
3. Size exclusion chromatography could be used to separate proteins of different sizes from the mutants of interest. Additionally, this step may provide information about the association state of Nap1.

## 4.2 In Vitro Binding Assays

The initial goals of performing GST pull-down assays was to determine if there was a difference in affinity of the Nap1 mutants for histones and determine if mutants defective for eviction were also defective for histone binding. Interestingly, results of GST pull-downs using GST-Nap1 deletion mutants in which histone octamer was added showed that the Nap1 deletion mutants have a different affinity for H2A/H2B dimer than they do for H3/H4 tetramer. The Nap1 deletion mutants show a decreased affinity for H3/H4 compared to wild type, with a significantly decreased affinity for H2A/H2B.

Previous published studies have shown that when GST pull-downs are performed with GST-Nap1, the affinity of Nap1 for H2A/H2B compared to H3/H4 changes depending on whether the Nap1 is present with dimer and tetramer separately or together as octamer [73]. When presented with dimer and tetramer, Nap1 has an equal affinity for both. When performed with an equal molar mixture of dimer and tetramer, Nap1 preferentially binds H3/H4 tetramer [73]. Although this pattern was not observed for full length Nap1 in our reactions, all of the Nap1 mutants expressed this binding pattern. Performing binding assays to compare dimer and tetramer may produce different for the binding capability of the Nap1 mutants. In this experiment, tetramer would be used as a control for dimer binding. It is possible that one or more of the mutants may not bind H2A/H2B even in the absence of H3/H4. (For example: if the C-terminal deletions are unable to interact with H2A/H2B, this would suggest that the



deletion affects H2A/H2B disassembly and could explain why the mutants are defective for nucleosome disassembly).

#### **4.3 Mutational Analysis of Full Length hNap1**

Because disruption of the oligomerization  $\beta$ -hairpin affects the nucleosome disassembly capability of Nap1, this suggests that the oligomeric form of Nap1 may be required to disassemble nucleosomes from the HTLV-1 promoter. To test this hypothesis point mutations in the  $\beta$ -hairpin of Nap1 could be made to prevent formation of the hydrogen bonds that participate in  $\beta$ -sheet formation in the oligomeric form of Nap1. A deletion mutant that replaces the  $\beta$ -hairpin with a flexible linker that would not support oligomerization could also be designed to test this hypothesis. Size exclusion chromatography could also be performed on these Nap1 mutants compared to wild type to characterize the association state of the various Nap1 mutants.

#### **4.4 Nap1 Interaction with other HTLV-1 Associated Proteins**

Current coimmunoprecipitation studies in our laboratory show that Nap1 and Tax interact in vivo. There is also evidence that Nap1 and p300 interact [59, 148, 149]. GST pull-downs using the Nap1 deletion mutants with p300 and Tax could aid in determining if the Nap1 deletion mutants are defective for nucleosome disassembly are also unable to bind another protein in the eviction reaction (i.e. p300 or Tax). If we find the mutants defective for histone eviction are also unable to interact with another protein in the eviction reaction, this would suggest that this interaction is critical for nucleosome disassembly.

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